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THE EFFECTS OF PRE-IRRADIATION HEAT INACTIVATION OF ENZYMES ON PALATABILITY OF BEEF AND PORK[a,b,c]

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(Manuscript received November 28, 1959)

Evidence has accumulated showing that the enzymes present in irradiated meats must be inactivated to prevent objectionable flavor changes during storage *(2, 3, 8)*. Although the enzymes in meat are partially inactivated by radiation, extremely high dosages are required for complete inactivation as shown in studies conducted by Doty and Wachter *(2)* and by Drake *et al (3)*. It has been established that meat contains a mixture of enzymes *(4, 9)* with different specificities, but in common with most other enzymes can be inactivated by heat treatment *(7)*. Thus, application of heat not only would prevent proteolysis but would produce a ready-to-eat meat item suitable for combat-type rations. Although many of the properties of the proteolytic enzymes isolated from meat tissues are known, these properties have been determined on isolated components and may not hold true for the enzymes *in situ*.

Consequently, this study was undertaken to ascertain the effects of various pre-irradiation enzyme inactivation temperatures upon acceptability, texture, pH and amino nitrogen changes during progressively longer periods of storage.

EXPERIMENTAL PROCEDURE

Meat selection and preparation. Boneless pork loins were cut into chops ¾ inch in thickness and randomly divided into 6 groups for different heat inactivation treatments. The *Longissimus dorsi* muscle was stripped from U. S. Choice wholesale beef ribs and cut into steaks 1 inch in thickness. The steaks were then assigned to 6 groups at random.

Enzyme inactivation. Both the steaks and chops were placed in a controlled smokehouse at 100% relative humidity and heat inactivated according to the holding times and temperatures recorded in Table 1. Internal temperatures and holding times were varied by using an external (smokehouse) temperature of either desired internal meat temperature + 10° F or 212° F until the specified internal temperature was achieved. When the desired internal temperature was reached, the external temperature of the smokehouse was reduced to the same point and held for the specified holding times. As soon as the holding times had elapsed, the meat was removed and immediately placed in a freezer at −20° F for 30–40 min to reduce the temperature. A rapid drop in temperature resulted but freezing did not occur in the length of time the meat was held in the freezer.

Packaging, irradiation, and storage. After cooling, the steaks and chops were sealed in No 2 cans under 28 inches of vacuum. They were then frozen at −20° F overnight and shipped in dry ice to Argonne National Laboratory, Lamont, Illinois. Irradiation was with a gamma source to a level of 5×10^6 rads. Two unirradiated

[a] Journal Article 2537 Michigan Agricultural Experiment Station, East Lansing.
[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been approved for publication.
[c] Presented at 19th Annual Meeting of the Institute of Food Technologists, Philadelphia, Pa., May 19, 1959.

TABLE 1

Temperatures of enzyme inactivation with holding times for beef steaks and pork chops

Internal temperature of meat	External cooking temperature [1]	Holding time at internal meat temperature [2]
° F	° F	° F
145 or 150 [3]	155 or 160 [3]	16 min
160	170	5 min
170	180	30 sec
145 or 150 [3]	212	16 min
160	212	5 min
170	212	30 sec

[1] Temperature of smokehouse until internal temperature was achieved.
[2] External temperature was lowered to coincide with the internal temperature of the meat once the desired temperature was achieved and the meat was maintained at the temperature for the specified holding times.
[3] The lower temperature was used for beef steaks and the higher for pork chops.

samples from each group were held in our laboratory in the frozen condition, with one to be used for chemical analysis and the other for taste panel evaluation.

After the meat was returned to our laboratory following irradiation, it was thawed at 36° F and stored at 72° F until removed for analysis. Storage was for 0, 3, 6 and 12 months.

Preparation and panel presentation. Cans for the panel were opened and the samples were placed in 6 inch loaf tins. They were then heated for 40 min in a 300° F electric oven. Coded samples were presented to an 18-member panel who rated them on the 9-point hedonic scale *(6)*.

Amino Nitrogen and chemical analysis. Amino nitrogen was determined with the Van Slyke apparatus on a slurry of 1 g of meat in 10 ml of solvent. Water was used as the solvent for beef but was not suitable for pork due to the higher fat content. Therefore, dioxane was selected as the solvent for pork after trying several others. Kjeldahl nitrogen was determined according to the A.O.A.C. procedure *(3)*, and amino nitrogen was calculated as the percentage of total nitrogen. The pH was measured on a meat-water slurry.

Texture ratings. Samples prepared for taste panel evaluation were observed for texture and the observations were recorded.

RESULTS

Panel ratings. Panel scores for unirradiated beef steaks were not significantly affected by the method of achieving enzyme inactivation (Table 2). However, there was a highly significant difference between the unirradiated and irradiated steaks prior to storage. In agreement with previously reported results *(5)*, there was a marked increase in panel score as storage of irradiated beef was extended from 0 to 3 months but a decline in acceptability occurred between 3 and 6 months.

Differences in panel scores between beef steaks inactivated at high or low external temperatures were not significantly different during the first 6 months of storage. However, after 12 months' storage the steaks treated with the higher inactivation temperature were preferred by the panel ($P = <.01$). Panel results indicated that the steaks inactivated at the higher external temperature were more stable during storage, which may have been due to incomplete enzyme inactivation at the lower temperatures.

With pork chops, the inactivation treatments did not result in any significant differences in acceptability of the unirradiated samples, but there was a highly significant difference between the irradiated and unirradiated chops prior to storage (Table 3). After 3 months' storage, panel ratings were higher than for the similarly irradiated chops tested at 0 months. Acceptability scores were lower at 6 months' storage, although they still tended to be higher than for the irradiated samples tested at 0 months. The mean panel scores for the chops inactivated at the higher external temperature (212° F) were lower ($P = <.01$) than the corresponding means for those inactivated at the lower external temperature (center meat temperature, + 10° F).

Percentage of amino nitrogen. A summary of the amino nitrogen changes in beef is given in Table 4. The percentage of amino nitrogen did not differ significantly be-

TABLE 2

The effects of pre-irradiation enzyme inactivation temperatures on panel scores of beef steaks

External inactivation temperatures	Internal meat temperature	Unirradiated [1]	Length of storage [2]				
			0 months	3 months	6 months	12 months	Means
155° F	145° F	6.3	2.1 [3]	4.0	4.0	3.2	3.3
170° F	160° F	6.2	3.7	4.4	4.7	3.9	4.2
180° F	170° F	6.3	4.1	6.7 [4]	5.4	4.4	5.2
Mean of low temp. inact.		6.3	3.3	5.0	4.7	3.9 [5]	4.2
212° F	145° F	5.8	4.5	4.6	4.2	4.8	4.5
212° F	160° F	6.2	3.8	5.6	4.7	4.9	4.7
212° F	170° F	6.1	4.3	5.4	5.0	5.4	5.1
Mean of high temp. inact.		6.1	4.2	5.2	4.6	5.1 [5]	4.8
Mean of all samples		6.2 [6]	3.7 [6]	5.1 [7]	4.7	4.5	4.5

[1] Tested only at 0 months' storage.
[2] All samples irradiated at 5×10^6 rads.
[3] All samples preferred over this sample at either 1% or 5% levels.
[4] At 3 months' storage, this sample, preferred over all other samples at either 1 or 5% level.
[5] At 12 months' storage, the mean of the samples inactivated at the higher temperature (212° F) was preferred ($P = <.01$) over the samples inactivated at the lower temperature.
[6] At 0 months' storage the mean for the unirradiated samples was preferred at 1% level over the mean for the irradiated samples.
[7] Mean for all samples at 3 months' storage preferred over mean for all irradiated samples at 0 months at 5% level.

TABLE 3

The effects of pre-irradiation enzyme inactivation temperature upon panel scores of pork chops

External inactivation temperature	Internal meat temperature	Unirradiated [1]	Length of storage [2]				
			0 months	3 months	6 months	12 months	Means
160° F	150° F	5.8	5.6	5.8	4.7	4.6	5.2
170° F	160° F	6.9	4.7	6.3	5.7	5.1	5.5
180° F	170° F	6.9	5.9	6.1	5.9	4.9	5.7
Mean of low temp. inact.		6.5	5.4 [3]	6.1	5.4	4.8	5.4 [4]
212° F	150° F	6.2	4.1	5.6	5.1	4.8	4.9
212° F	160° F	6.6	3.9	4.9	5.1	4.7	4.6
212° F	170° F	6.9	3.9	5.8	5.6	5.3	5.2
Mean of high temp. inact.		6.6	4.0	5.5 [5]	5.3	4.9	4.9 [4]
Mean of all samples		6.6 [6]	4.7 [6]	5.8	5.3	4.9	5.2

[1] Tested only at 0 months' storage.
[2] All samples irradiated at 5×10^6 rads.
[3] Mean of low temperature inactivated samples preferred at 1% level over all samples inactivated at 212° F. and tested at 0 months storage.
[4] Over-all mean for samples inactivated at the lower temperatures were preferred by the panel ($P = <.01$) over the samples inactivated at the higher temperature.
[5] At three months' mean for low temp. inact. samples contained significantly higher ($P = <.01$) perferred over the mean for those inactivated at 212° F.
[6] Difference between all irradiated and unirradiated samples at 0 days was significant at 1% level at 0 months' storage.

tween the unirradiated samples regardless of the temperatures used for enzyme inactivation. Irradiation tended to result in an increase in amino nitrogen, although it was very slight at the higher external temperature. The samples inactivated at the lower external temperatures (center temperature, + 10° F) contained a significantly greater percentage of amino nitrogen at the end of each storage period except at 12 months. The increase was especially evident with the samples inactivated by heating to an internal temperature of 145° F at an external temperature of 155° F. There was a general stepwise increase

TABLE 4

Amino nitrogen changes associated with enzyme inactivation temperatures and length of storage for beef steaks*

External inactivation temperature	Internal meat temperature	Unirradiated [1]	Length of storage [2]				Means
			0 months	3 months	6 months	12 months	
155° F	145° F	7.6	8.4	9.5	11.5	11.4	10.2
170° F	160° F	8.1	8.9	8.2	8.8	9.1	8.7
180° F	170° F	7.6	8.0	8.4	8.4	8.9	8.4
Mean of low temp. inact.		7.8	8.4 [3]	8.7	9.6	9.8	9.1 [4]
212° F	145° F	7.9	7.9	7.8	7.7	8.2	7.9
212° F	160° F	7.2	7.9	7.8	7.7	9.5	8.1
212° F	170° F	7.8	7.2	7.3	7.2	8.2	7.5
Mean of high temp. inact.		7.6	7.7 [3]	7.5 [5]	7.5 [6]	8.6	7.8 [4]
Mean of all samples		7.7	8.0	8.2	8.6	9.2	8.5

* Percentage of amino nitrogen.
[1] Tested only at 0 months' storage.
[2] All samples irradiated at 5×10^6 rads.
[3] At 0 months' mean of low temp. inact. samples contained significantly higher ($P = <.01$ percentage of amino nitrogen than the higher temperature of inactivation.
[4] The over-all mean for the low temperature inactivated samples contained a greater amount of amino nitrogen ($P = <.01$) than the mean of the high temperature inactivated sample.
[5] At 3 months' mean for low temperature inactivated sample contained significantly higher ($P = <.01$) percentage of amino nitrogen.
[6] At 6 months, mean for low temp. inactivation samples contained significantly higher percentage of amino nitrogen ($P = <.01$) than the higher temperature of inactivation.

in percentage of amino nitrogen as length of storage was prolonged. Thus, results indicate that inactivation of the enzymes was more complete at an external temperature of 212° F than at the lower temperature (center meat temperature, + 10° F).

With unirradiated pork chops, the temperatures of enzyme inactivation did not significantly influence the percentage of amino nitrogen (Table 5). However, irradiation resulted in a significant increase in percentage of amino nitrogen. There was no significant difference between the high and low external temperatures of enzyme inactivation, except at 3 months' storage, when the percentage of amino nitrogen was significantly higher for the mean of the low external temperature of enzyme inactivation.

TABLE 5

Amino nitrogen changes associated with enzyme inactivation temperatures and length of storage for pork chops

External inactivation temperature	Internal meat temperature	Unirradiated [1]	Length of storage [2]				Means
			0 months	3 months	6 months	12 months	
160° F	150° F	4.2	5.5	6.9	8.6	5.7	6.9
170° F	160° F	4.7	6.1	6.4	7.1	6.2	6.5
180° F	170° F	4.3	5.5	6.4	6.2	5.4	5.9
Mean of low temp. inact.		4.4	5.7	6.6 [3]	7.3	5.8	6.3
212° F	150° F	3.6	6.1	5.1	7.9	6.5	6.4
212° F	160° F	4.7	6.4	5.3	7.2	7.1	6.5
212° F	170° F	4.3	5.9	5.4	7.0	5.2	5.9
Mean of high temp. inact.		4.2	6.1	5.3 [3]	7.4	6.2	6.2
Mean of all samples		4.3 [4]	5.9 [4]	5.9	7.3	6.0	6.3

[1] Tested only at 0 months' storage.
[2] All samples irradiated at 5×10^6 rads.
[3] Mean of low temperature inactivated samples at 3 months contained a greater percentage of amino nitrogen ($P = <.05$) than those inactivated at the higher temperature.
[4] Difference between irradiated and unirradiated samples was highly significant.

Thus, the temperatures of enzyme inactivation appeared to be adequate to prevent further enzymic degradation.

Texture observations. There was no apparent difference in texture between any of the unirradiated samples of either beef or pork with all samples being firm. Similarly, the method of enzyme inactivation did not appear to influence the texture of the samples observed after irradiation, but all samples were slightly mushy after irradiation. After 3 and 6 months' storage, texture changes were apparent with various samples. Although the consistency varied between samples, it did not appear to follow any established pattern. However, a close relationship between texture and panel scores was evident as storage proceeded, with the mushy and crumbly samples being rated down while the firm appearing samples were rated higher. Thus, texture appeared to be associated with irradiation and length of storage but did not seem to be related to temperature of enzyme inactivation.

Changes in pH. With beef at 0 months' storage for both irradiated and unirradiated steaks, the mean pH for the low temperatures of inactivation was higher ($P = <.01$) than the corresponding values for the high external temperature. Although the differences in pH were not significant after 3, 6 or 12 months' storage, the over-all mean for the low temperature inactivated steaks was higher ($P = <.01$) than the corresponding value for the steaks inactivated at 212° F. The average pH for all unirradiated samples and for all irradiated beef steaks stored for 12 months was lower than for the corresponding means for the irradiated steaks stored for 0, 3 or 6 months ($P = <.01$), although the reason for the lower value after 12 months' storage is not apparent.

For pork, the over-all mean for pH at the low temperature of inactivation was significantly lower ($P = <.01$) than at the higher temperature, which is the reverse of that found with beef. The reason for variation from the beef steaks is not readily apparent, but may be due to a species difference.

DISCUSSION

Results show that the beef steaks inactivated at the lower temperatures of inactivation (center meat temperature, +10° F) were rated lower by the panel than those inactivated at the higher temperature (212° F). The fact that the level of amino nitrogen was higher at the lower temperatures of inactivation indicated that the enzymes were not completely inactivated by the milder heat treatment and that proteolysis continued. Further evidence was obtained to support this theory by the fact that there was a stepwise increase in amino nitrogen as length of storage was increased. It was also noted that the magnitude of the increase in amino nitrogen was greater at each successive lowering of the external inactivation temperature.

With pork, there was no apparent difference in the amount of amino nitrogen between samples inactivated at the different external temperatures, although the samples inactivated at the lower temperatures were preferred by the panel. Thus, it appears that the heat treatments were all adequate to destroy the enzymes. It should be pointed out that the lowest temperature of inactivation used for the pork was 5 degrees higher than with the beef, which may give more complete enzyme inactivation. However, the fact that proteolysis was not evident at even the lowest temperature of inactivation tends to discount this possibility. The differences observed in panel score in favor of the lower temperatures of inactivation would appear to be due to greater juiciness or other palatability factors associated with the milder heat treatment.

The lower scores associated with the loss of texture upon storing irradiated meat indicates this is one of the serious problems. Irradiation appears to be a contributing factor to the loss of texture in precooked irradiated meat, but the texture becomes even poorer as storage is prolonged. It has been observed

that texture loss is not a serious problem in irradiated fresh meats and that consistency is good, even after long periods of storage. This would suggest that alteration of pH or the use of chemical inhibitors could be used on fresh meats prior to irradiation to control enzymatic degradation and that the changes in texture encountered with precooking as a means of enzyme inactivation could thereby be circumvented.

SUMMARY

A study was conducted to ascertain the influence of various pre-irradiation enzyme inactivation temperatures upon flavor, texture and amino nitrogen changes during progressively longer periods of storage. Results of panel evaluation, texture observations and percentage of amino nitrogen indicated that the higher external temperatures more effectively destroyed the enzymes in beef. However, for pork the lowest temperature of inactivation appeared to prevent proteolysis and the resultant loss of acceptability. Observations would indicate that prevention of texture changes is a separate problem associated with the storage of irradiated heat treated beef and pork.

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THE EFFECTS OF PRE- AND POST-ENZYME INACTIVATION STORAGE ON IRRADIATED BEEF AND PORK ROASTS[a,b]

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(Manuscript received December 7, 1959)

Irradiated fresh meats can be stored for long periods of time without visible change, but flavor deterioration occurs as storage progresses. The alteration in flavor, which appears to be due to enzymatic degradation, can be prevented by heat inactivation of the enzymes prior to irradiation *(3, 4, 7)*. Although the flavor of the meat improves, as storage progresses, for short periods of time, the texture becomes poorer when storage is prolonged *(4)*.

A number of studies *(2, 4, 5, 6)* have been made on the acceptability and chemical composition of meat that has been enzyme inactivated prior to storage but the effects of post-irradiation enzyme inactivation followed by storage are virtually unknown. Therefore, this study was designed to ascertain the effects of both pre- and post-enzyme inactivation storage upon acceptability, texture, amino nitrogen, and pH of irradiated beef and pork roasts.

EXPERIMENTAL

For the beef work, the *Longissimus dorsi* muscle was stripped from U. S. Choice wholesale beef ribs and cut into roasts to fit into a No 2 can. Boneless pork loins were also cut and fitted into a can in the same manner. The cans (C-enamel for pork) were then sealed under 28 in of vacuum. A number of cans were fitted with thermocouples, which were subsequently attached to a recording potentiometer to give temperature records during heat-inactivation of the enzymes.

Enzyme inactivation. The enzymes in the beef roasts were heat inactivated by injection of live steam into an air conditioned smokehouse to give a temperature of 100° C (212° F) until the internal temperature of the roasts reached 74° C (165° F). The pork roasts were enzyme inactivated by the same procedure, except that the external smokehouse temperature was held at 79° C (175° F) until the internal meat temperature reached 74° C (165° F). Inactivation was accomplished after either 0, 4, 8, 16, 32 or 64 days' storage.

Irradiation, shipping, and storage. After sealing, the cans were frozen, packed in dry ice and shipped to Argonne National Laboratory, where they were irradiated to a level of 5 megarads with a Co_{60} source. On return to our laboratory, the cans were placed at 24° C (76° F) and held until removed for enzyme inactivation and/or testing. At the end of 0, 4, 8, 16, 32 or 64 days' storage in the raw state, 4 samples were removed and enzyme inactivated as described previously. Two samples were then used for panel testing and chemical analysis; the remaining two were returned to the same storage conditions, until a total storage time of 90 days had elapsed. They were then subjected to the same tests.

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[a] Journal Article 2527 Michigan Agricultural Experiment Station, East Lansing.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 1064 in the series of papers approved for publication.

Panel testing. After heating to an interal temperature of 66° C (150° F), the meat was cut into small samples, coded, and presented to an 18-member untrained panel, who rated the samples on the 9-point hedonic scale.

Chemical analysis. At the time the roast was prepared for the taste panel, a duplicate sample was removed and either analyzed immediately or held at −29° C (−20° F) until analysis. The entire roast was ground through the fine plate of a Hobart food chopper 5 times. A 1:10 meat-water slurry was prepared in a blender. Determination of pH was made on the slurry with a Beckman pH meter, and total nitrogen was measured by the Kjeldahl-Gunning method *(2)*. With the beef, amino nitrogen was determined on the meat-water slurry by the Van Slyke method *(1)*. However, the consistency of pork fat required the use of a different solvent, so dioxane was used. Consequently, 1 g of meat was blended in 10 ml of dioxane and amino nitrogen was determined by the Van Slyke procedure *(1)*. Amino nitrogen was calculated on the basis of the percentage of total nitrogen.

Texture. On preparation of the roasts for presentation to the taste panel, the texture was observed and recorded for each sample.

RESULTS

Amino nitrogen. A summary of the percentage of amino nitrogen present in the roasts after various periods of storage is presented in Table 1. With beef analyzed at the time of enzyme inactivation, there was an irregular increase in amino nitrogen during the first 8 days of storage, but as storage proceeded further there was a marked and consistent rise. Thus, results show that enzymatic breakdown continued in the raw irradiated beef for storage periods up to at least 64 days. This is in agreement with the work of previous investigators, which has shown that amino nitrogen continues to increase until the enzymes are inactivated. Beef roasts inactivated and then stored until a total storage time of 90 days had elapsed generally showed no further increase in amino nitrogen. There were a few exceptions, which were probably associated with poor sampling techniques or perhaps with incomplete destruction of the enzymes; in general, however, enzyme inactivation appeared to prevent further proteolysis.

With pork stored through 8 days, there was little apparent increase in percentage of amino nitrogen, but beyond 8 days' storage there was a marked and consistent rise. Similar to the beef, there was little change in percentage of amino nitrogen during additional storage following enzyme inactivation. Thus, it appears that heat-inactivation of enzymes should be carried out sometime prior to 16 days' storage in order to minimize enzymatic changes.

pH. Although there was considerable variation in pH within each kind of roast, the differences were not statistically significant. Thus, pH did not appear to be influenced by differences in treatment, nor was there any evident association between pH and either amino nitrogen or panel scores.

Acceptability. Panel scores are presented in Table 2. With beef, there tended to be a gradual decline in panel score as the length of storage prior to heat inactivation of the enzymes was increased. After 64 days storage prior to inactivation, the panel rating was significantly lower than all samples except those held for 4 days. The reason for the extremely low values at 4 days cannot be explained. Although results are variable, there appeared to be little change in panel score following heat inactivation of the enzymes.

With pork, there was a significant decline in panel score, when the roasts were stored in the raw irradiated state. The drop in ratings occurred somewhere between 32 and 64 days' storage. The pork roasts stored after enzyme inactivation received a higher panel score ($P = <.01$) than those tested without subsequent storage. This indicates that the additional storage generally improved the flavor, which is not especially surprising, since previous work *(4)* has shown that storage after irradiating and cooking resulted in improvement in flavor.

Texture. Samples tested at the time of inactivation were firmer than after additional storage. The samples remained in a fairly good physical condition while held in the raw irradiated state until 32 days of storage had elapsed. However, at 64 days both the

TABLE 1

The effects of pre- and post-enzyme inactivation storage periods upon percentage of amino nitrogen in irradiated beef and pork roasts

Treatment	Length of storage prior to enzyme inact.	Beef roasts			Pork roasts		
		Samples tested at time of inact.	Samples tested after 90 days total storage	Means	Samples tested at time of inact.	Samples tested after 90 days total storage	Means
Frozen-unirradiated control	90 days (frozen)	.....	7.9	7.9	.....	.....	.....
Pre-irradiation enzyme inact	0 days	6.8	7.8	7.3	5.6	6.4	6.0
Post-irradiation enzyme inact	0 days	6.8	7.3	7.1	5.6	5.0	5.3
Post-irradiation enzyme inact	4 days	8.4	7.9	8.1	5.9	5.5	5.7
Post-irradiation enzyme inact	8 days	7.9	9.2	8.6	5.4	6.5	6.0
Post-irradiation enzyme inact	16 days	10.9	10.7	10.8[1]	7.0	7.3	7.2[2]
Post-irradiation enzyme inact	32 days	11.3	13.3	12.3[3]	8.7	8.1	8.4[4]
Post-irradiation enzyme inact	64 days	14.0	14.8	14.4[5]	11.4	12.2	11.8[6]
Means		9.4	10.1	9.8	7.1	7.3	7.2

[1] Beef stored 16 days contained a greater percentage of amino nitrogen than all samples stored for shorter periods of time ($P = <.01$).
[2] Pork stored 16 days before irradiation had a greater amount of amino nitrogen ($P = <.05$) than all post-irradiation samples stored for a shorter period of time.
[3] Beef held 32 days before inactivation contained a significantly greater ($P = <.05$) percentage of amino nitrogen than the sample inactivated at 16 days and a greater amount than all samples stored for less than 16 days ($P = <.01$).
[4] Pork roast inactivated at 32 days contained a greater amount of amino nitrogen than all samples stored a shorter period of time ($P = <.01$).
[5] Beef held 64 days before inactivation contained a greater percentage of amino nitrogen than the sample stored 32 days ($P = <.05$) and a greater amount ($P = <.01$) than all other samples.
[6] Pork roast inactivated at 64 days contained a greater percentage of amino nitrogen ($P = <.01$) than all samples held a shorter period of time.

TABLE 2

The effects of pre-enzyme and post-enzyme inactivation storage periods upon panel scores of irradiated beef and pork roasts

Treatment	Length of storage prior to enzyme inact.	Beef roasts			Pork roasts		
		Samples tested at time of inact.	Samples tested after 90 days total storage	Means	Samples tested at time of inact.	Samples tested after 90 days total storage	Means
Frozen-unirradiated control	90 days (frozen)		7.7	7.7 [1]		6.7	6.7 [2]
Pre-irradiation enzyme inact	0 days	4.9	5.2	5.1 [3]	4.7	5.6	5.2
Post-irradiation enzyme inact	0 days	4.4	4.9	4.7	5.2	6.0	5.6
Post-irradiation enzyme inact	4 days	3.6	4.4	4.0	5.1	5.2	5.1
Post-irradiation enzyme inact	8 days	5.1	5.1	5.1 [3]	4.2	5.0	4.9 [4]
Post-irradiation enzyme inact	16 days	4.8	4.2	4.5	4.7	5.4	5.1
Post-irradiation enzyme inact	32 days	4.8	4.7	4.8	5.3	5.2	5.2
Post-irradiation enzyme inact	64 days	3.3	3.7	3.5 [5]	3.6	4.6	4.1 [6]
Means [7]		4.4	4.6		4.7 [8]	5.3	

[1] Unirradiated control preferred over all samples at $P = <.01$.
[2] Unirradiated sample preferred at either 1 or 5% level over all samples except those inactivated prior to irradiation.
[3] Means for samples inactivated before irradiation and after 8 days storage preferred over samples inactivated after 4 days storage ($P = <.05$) and over samples heat inactivated after 64 days ($P = <.01$).
[4] Mean of samples inactivated at 8 days rated lower ($P = <.05$) than mean for samples inactivated at 0 days.
[5] All samples preferred over those inactivated at 64 days at either the 1 or 5% level except for the samples heat inactivated at 4 days after irradiation.
[6] Mean of 64 days inactivated samples significantly ($P = <.05$) lower than all other means except 8 day inactivated sample.
[7] Mean does not include unirradiated control.
[8] Mean difference between samples tested at time of heat inactivation and after 90 days storage significant at 1% level.

pork and beef were mushy and of poor texture. Furthermore, there appeared to be a tendency for low panel scores to be associated with poor texture.

DISCUSSION

Earlier work on inactivation has generally been carried out in an open container with sealing of the cans after cooling. This procedure has been followed to avoid the typical canned meat flavor, which is commonly found in canned meats subjected to the high temperatures necessary for heat sterilization. However, with the temperatures used for enzyme inactivation in the study, there were no adverse effects evident from inactivating the enzymes in sealed cans. This is apparent when one notes that pre-irradiation enzyme inactivated samples did not differ significantly from post-irradiation enzyme inactivated samples at 0 days storage (Table 2) in panel scores. Furthermore, there were no complaints from panel members on canned meat flavor with any samples. Thus, heat inactivation of the enzymes would appear to be entirely feasible.

The combined results of the taste panel, amino nitrogen percentage, and texture of the roasts all indicate that enzyme inactivation can be satisfactorily delayed for 8 days with beef and up to 16 days for pork. It should be recognized, however, that conditions of storage different from those used in this study would probably require different recommendations.

It was hoped that inactivation of the enzymes after storage would result in improvement of texture over samples inactivated prior to irradiation. However, tissue breakdown still occurred and after 90 days' total storage practically all irradiated samples showed considerable crumbliness or mushiness.

SUMMARY

The effects of delaying enzyme inactivation upon panel scores, texture ratings, amino nitrogen content and the pH of irradiated beef and pork are reported. It was found that the raw irradiated roasts could be stored at 24° C (76° F) for periods up to 16 days before enzyme inactivation without adverse effects upon panel acceptability. Although proteolysis, as measured by the increase in amino nitrogen, occurred as storage of the raw irradiated roasts was prolonged, the increase was not marked until after 8 days. The increased protein breakdown appeared to be responsible for the poor acceptability and mushy texture of roasts held 32 days or longer before enzyme inactivation. Although the method of heat inactivation used appeared to be adequate to prevent further protein degradation, the texture continued to become poorer with post-enzyme inactivation storage. Thus, the texture problem on storage seemed to be independent of protein breakdown. No clear-cut effects of treatment upon pH was evident, nor was there any evident association between pH and amino nitrogen, texture or panel scores.

Acknowledgments

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THE EFFECT OF MATURITY AND STORAGE ON PHENOLIC CONTENT, ENZYMATIC ACTIVITY AND DISCOLORATION OF POTATOES[a]

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Discoloration of raw potatoes, an abnormal physiological condition which occurs when potatoes are bruised, is one of the most serious and costly problems of the potato industry. If the mechanism responsible for this type of discoloration could be clearly defined, perhaps methods of controlling or preventing discoloration might be developed.

Some factors believed to influence the tendency of tubers to discolor are maturity, storage duration, and variety *(14, 18)*. Susceptibility to blackening was observed to increase with increasing maturity and storage duration. Potato discoloration has been associated with the action of polyphenol oxidase on the phenolic constituents of the potato resulting in the formation of the black pigment, melanin. The enzymatic oxidation of mono- and diphenols catalyzed by polyphenol oxidase produces the black pigment, melanin *(12)*.

The role of polyphenol oxidase in the metabolism of the potato has not been clearly defined. Early workers associated this enzyme with terminal oxidation in the tuber *(1, 2, 3)*, but more recent studies have indicated that cytochrome oxidase activity is high enough to account for the total respiration of the tuber *(7, 17, 19)*. Joslyn and Ponting *(11)* pointed out that cytochrome oxidase reacts with certain phenols to convert them into pigmented compounds and under certain conditions may be involved in darkening. Relatively high concentrations of phenolic substances such as tyrosine, chlorogenic acid and caffeic acid, which could serve as substrates for enzymes involved in discoloration, have been found in potatoes *(4, 6, 9, 10)*. Varietal differences in phenolic content have been reported by several workers *(5, 6, 8)*. Craft, Siegelman and Butler *(6)* found that the total phenolic content in two varieties of potatoes did not change significantly after storage. Previous work in this laboratory, using time of harvest following planting as the criterion for expressing maturity, showed highest phenolic content in tubers from the second of three harvests.

In the present study, two varieties of potatoes known to differ in their tendency toward precooking discoloration were used. A study was made of the interrelationships of variety, maturity and storage duration on phenolic content, discoloration, cytochrome oxidase and polyphenol oxidase activities of potatoes.

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EXPERIMENTAL

Two varieties of potatoes, Pontiac which is resistant to, and Ontario susceptible to precooking discoloration, were grown near Ithaca, New York in each of two consecutive years, 1956 and 1957. Potatoes were planted so that soil variation would be randomly distributed among the plots and were harvested 7, 9, 11, 13, 15 and 17 weeks after planting in 1956, and 10, 13, and 17 weeks after planting in 1957. The potatoes varied in size from one inch in diameter in the early harvests to 5 inches or more in the later harvests. The criterion used for expressing maturity was the time of harvest in relation to planting date. Determination of phenolic content, discoloration, and of cytochrome oxidase and polyphenol oxidase activities were made at the time of harvest, and after 3, 7, 12 and 24 weeks of storage in 1956, and after 4, 8, 12 and 24 weeks of storage in 1957. All potatoes were stored at 40° F.

Determination of total phenols. Four tubers of each variety were selected at random for each extract used in phenol determinations. Three extracts were made for each harvest at each storage period in 1956, and two extracts at each storage period in 1957. A 50 g sample of the cortex tissue was blended with 150 ml of 95% ethanol for 5 min. The total extract was measured, filtered, and 35 ml of the filtrate used for phenol determination. Total phenolic constituents were determined by the method of Rosenblatt and Peluso *(16)* using tannic acid as the standard. Since this method is not specific for phenols but also includes ascorbic acid, corrections were made for ascorbic acid. Potato tissue was extracted with meta-phosphoric acid and analyzed for ascorbic acid by the *indophenol dye* method simultaneously with the analyses for total phenols.

Determination of enzyme activity. Three tubers of each variety were selected at random for each extract. Three extracts were made for each harvest at each storage period in 1956, and two extracts at each storage period in 1957. A 50 g sample of cortex tissue, taken longitudinally from the bud to stem end of the potatoes, was blended with 50 ml of 0.05 M sodium barbiturate for 1 min, and filtered through cheesecloth for 3 min. The filtrate was then centrifuged at $600 \times g$ for 3 min, and the supernatant liquid used for enzyme activity determinations. All reagents and containers were chilled before and during the preparation of the extract. Cytochrome oxidase and polyphenol oxidase activities were determined manometrically by a modification of the method described by Goddard and Holden *(7)*. For the determination of cytochrome oxidase, the main compartment of the Warburg flask contained 1 ml of 0.5 M phosphate buffer at pH 7.1 plus 0.6 ml of 2.25×10^{-4} M cytochrome *c*; the center well 0.2 ml of 2 N sodium hydroxide; one side arm 0.3 ml of the potato extract; the second side arm 0.8 ml of 6.82×10^{-2} M hydroquinone. Water was added to the main compartment to bring the total volume of the liquid in the flask to 3.2 ml. The side arm contents were added after a 5 min equilibration period at 30° C; stopcocks were closed, and readings taken every 10 min over a 1-hr period. Polyphenol oxidase activity was measured similarly using, in the 1956 study, 0.2 ml of 4.55×10^{-3} M catechol instead of cytochrome *c* and 0.2 ml of the potato extract and, in the 1957 study, 0.4 ml of 4.55×10^{-3} M catechol and .4-ml potato extract. Cytochrome oxidase and polyphenol oxidase activities were determined simultaneously on the same potato extract.

Determination of discoloration. Color measurements were made on 200 g of ground potato tissue using the Hunter Color Difference Meter. The standard gray tile used for setting the instrument had the values: Rd 39.0, a −1.1, and b −3.3. Only Rd values are reported here since these were most closely associated with the potato discoloration observed visually. Longree *(13)*, using potato patties, found that Hunter values were correlated with visual color scores.

Potatoes were peeled before they were ground for color measurements. Tissue from 4 potatoes was used for each slurry. Duplicate determinations were made on each lot of potatoes at each storage period.

In 1956 the entire tuber was sampled. Potatoes were sampled by cutting portions longitudinally from bud to stem end. A 200-g sample was ground in a Waring blender with 10 ml of distilled water for 5 min, the slurry filtered, and the residue allowed to stand at room temperature for 30 min before color determinations were made. In 1957 only cortex tissue was used, since this is the area known to discolor most readily following bruising. A 200-g sample was ground in a food grinder, using the "coarse" attachment, allowed to stand at room temperature for 20 min, then transferred to a plexiglas cup and color reflectance readings taken immediately.

RESULTS AND DISCUSSION

Cytochrome oxidase activity. The analysis of variance was applied to the data and the differences in cytochrome oxidase activity due to maturity, storage and variety were found to be significant at the 1% level. Cytochrome oxidase activity at the time of harvest is shown in Figure 1. In both years the cytochrome oxidase activity was lowest in the most mature potatoes. In the 1956 study the enzyme activity was inversely related to maturity, but this trend was less consistent in 1957 (Figure 1). The same maturity effect on enzyme activity was evident throughout storage (Figure 2). In both years cytochrome oxidase activity increased during the first three months of storage (Figure 3).

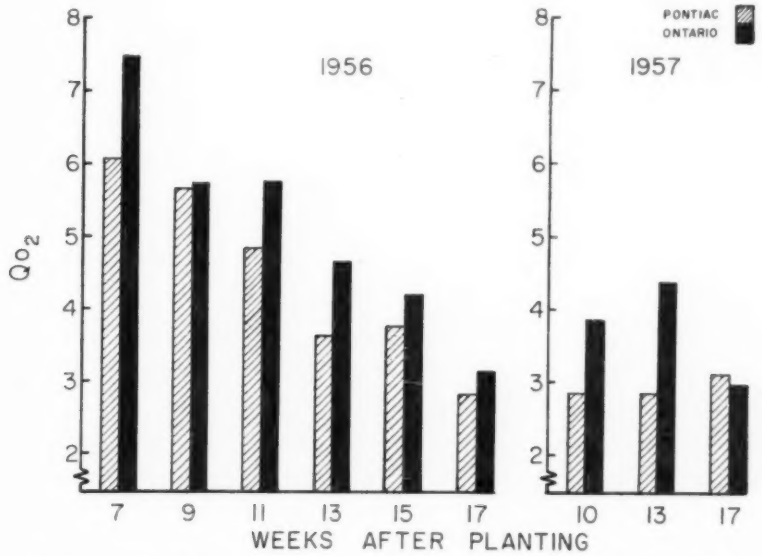

Figure 1. The effect of maturity on cytochrome oxidase activity of potatoes before storage.

(Q_{O_2}: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.) Each value plotted is the mean of 3 determinations on 9 potatoes in 1956 and 2 determinations on 6 potatoes in 1957.

Potatoes of the 1956 harvests showed a marked decrease in cytochrome oxidase activity during the first 3 weeks in storage, followed by a rapid increase in activity up to 3 months of storage. No decrease in cytochrome oxidase activity was observed, however, when the 1957 harvests were placed in storage. These differences in response to storage may have been due to differences in initial sampling, for in 1956 the potatoes were sampled immediately after they were brought from the field, whereas, in 1957, the potatoes were sampled a few days following harvest. Ontario potatoes were consistently higher in cytochrome oxidase activity than Pontiac potatoes.

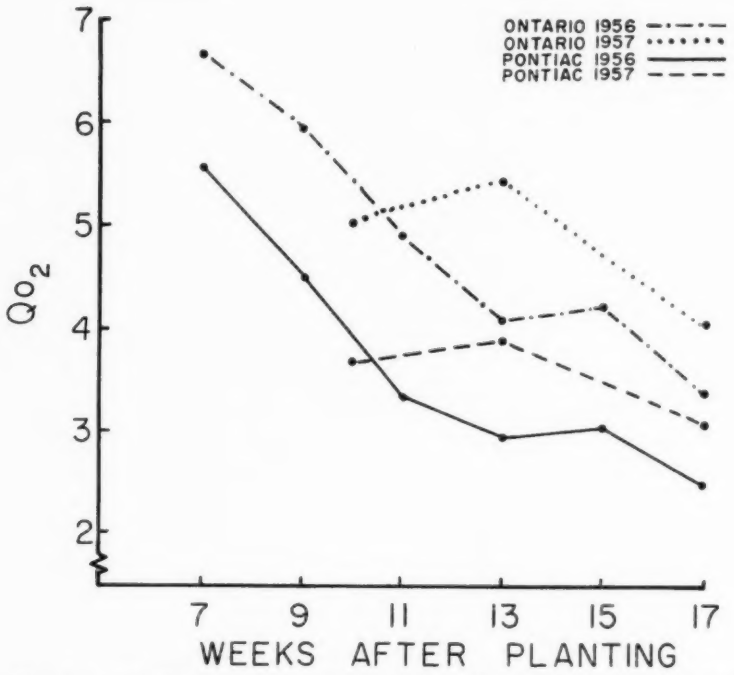

Figure 2. The effect of maturity on cytochrome oxidase activity of potatoes after storage.

(Q_{O_2}: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.) Data from the 5 storage periods are combined. Each value plotted is the mean of 15 determinations on 45 potatoes in 1956 and 10 determinations on 30 potatoes in 1957.

Polyphenol oxidase activity. Differences in polyphenol oxidase activity due to the effects of variety, maturity and storage were significant at the 1% level. Ontario potatoes were higher in polyphenol oxidase activity than Pontiac potatoes. In 1957, potatoes increased in polyphenol oxidase activity with increasing maturity, and in 1956 the last harvests had the highest polyphenol oxidase activity (Figure 4). The maturity effect continued to be evident throughout storage (Figure 5).

The effect of storage on polyphenol oxidase activity of potatoes was fairly consistent in the two-year study (Figure 6). In both years the enzyme activity decreased sharply during the first month of storage and rose only slightly during the remainder of the six-month storage period. At no time in storage did the enzyme activity reach the same level as that of potatoes which were freshly harvested. These findings are in agreement with the observation of Mulder *(15)* that polyphenol oxidase activity decreases as the tuber ages. In the present study the tendency for potato discoloration to increase with storage was apparently not associated with increased polyphenol oxidase activity.

Phenolic content. Differences in phenolic content of potatoes due to the

effects of maturity, storage, and variety were highly significant. Immature potatoes had a higher phenolic content than mature potatoes before storage (Figure 7). Phenolic content decreased with increasing maturity of the tuber in 1957, and although in 1956 there was no consistent trend with maturity, potatoes in the last three harvests had a lower phenolic content than did those of the first three harvests. The effect of maturity on the phenolic content of potatoes during storage is given in Figure 8. In 1956 and 1957 the highest phenolic concentrations were found in the most immature potatoes. A similar relationship between phenolic concentration and maturity was found in apples by Weurman and Swain *(20)*.

The effect of storage on phenolic content of tubers is shown in Figure 9. Before storage, the phenolic content of Pontiac potatoes was higher than Ontario potatoes, but after 6 months of storage the varietal differences were reversed, and Ontario potatoes were higher in phenolic substances. By the end of the six-month storage period in 1956, the Ontario variety had increased in phenolic content by 60% and the Pontiac by 36%. Similarly in 1957, the

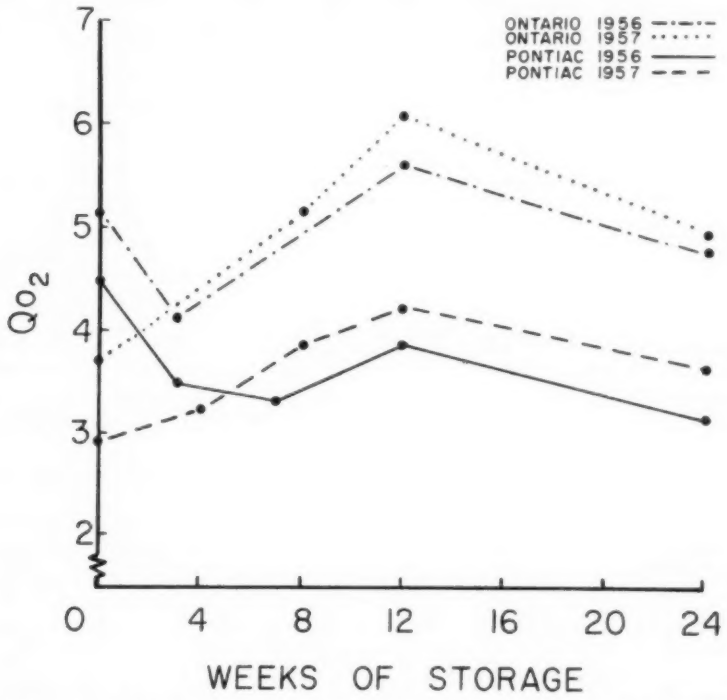

Figure 3. The effect of storage on the cytochrome oxidase activity of Pontiac and Ontario potatoes.

(Q_{O_2}: microliters of oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.) In 1956 data from 6 harvests are combined and in 1957 data from 3 harvests. Each value plotted is the mean of 18 determinations on 54 potatoes in 1956 and 6 determinations on 18 potatoes in 1957.

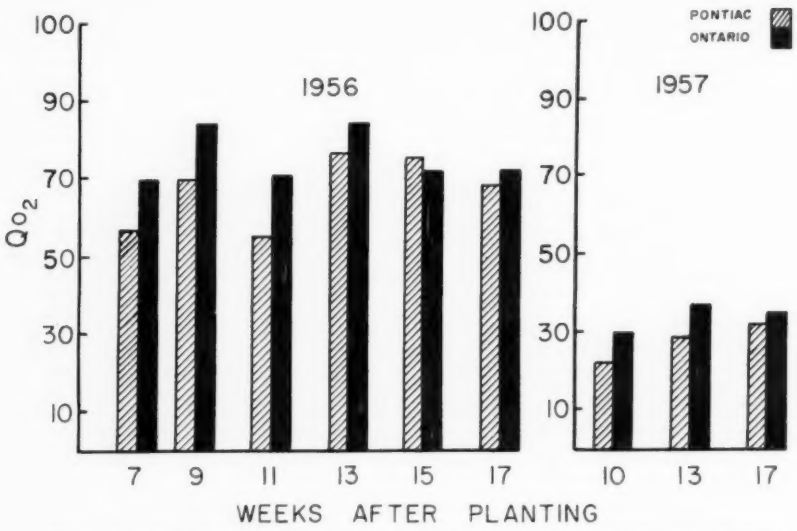

Figure 4. The effect of maturity on the polyphenol oxidase activity of potatoes before storage.

(Q_{O_2}: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.) In 1956 the system included .2 ml potato extract and .2 ml catechol, and in 1957 .4 ml potato extract and .4 ml catechol. Each value plotted is the mean of 3 determinations on 9 potatoes in 1956 and 2 determinations on 6 potatoes in 1957.

Ontario variety increased 78% and the Pontiac 25% in phenolic compounds.

Changes in phenolic content of potatoes after storage, as found in this study, are not in agreement with the findings of Craft *et al (6)* who reported no significant change in phenolic content of two varieties of potatoes stored for five months at 40° F. Differences in findings may be due to differences in potato varieties studied, or in the part of the tuber analyzed for phenolic content. Craft *et al* sampled the entire tuber; in this study only the cortex tissue was analyzed, since the cortex had been found to be the area highest in phenolic content *(4)*.

Discoloration. Differences due to the effects of maturity, storage and variety on potato discoloration were significant at the 1% level.

In both years Ontario potatoes in the last harvest showed the greatest tendency toward discoloration (Table 1). The effect of potato maturity on discoloration was not as pronounced after the potatoes were stored, but, generally, later harvests showed the greater tendency toward discoloration (Table 2). In both years discoloration increased with increasing storage duration (Table 3), and Ontario potatoes darkened more than Pontiac potatoes. These findings are in agreement with those of earlier workers *(14, 18)*.

Interrelationships of enzyme activities, phenolic content, and discoloration. The decrease in polyphenol oxidase activity during potato storage was accompanied by an accumulation of phenolic substances and an increased tendency toward discoloration. Immature potatoes, which had a relatively

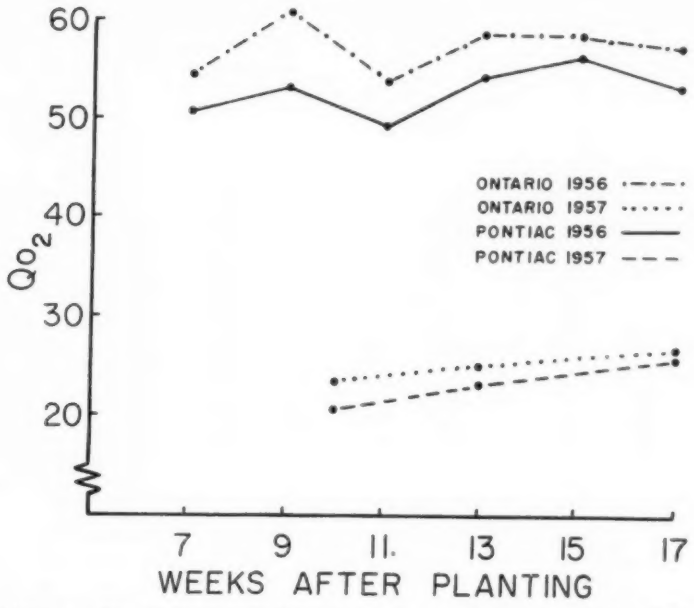

Figure 5. The effect of maturity and variety on polyphenol oxidase activity of potatoes after storage.

(Q_{O_2}: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.) The system in 1956 included .2 ml potato extract and .2 ml catechol and in 1957 .4 ml potato extract and .4 ml catechol. Data from all 5 storage periods are combined. Each value plotted is the mean of 15 determinations on 45 potatoes in 1956 and 10 determinations on 30 potatoes in 1957.

TABLE 1

Effect of maturity on potato discoloration before storage

Harvest	Weeks after planting	Variety	
		Pontiac	Ontario
1956		Rd [1]	Rd
1	7		
2	9	42.5	40.7
3	11	34.3	36.9
4	13	35.2	33.9
5	15	31.8	35.8
6	17	29.8	27.3
1957			
1	10	32.1	26.8
2	13	37.1	32.9
3	17	38.4	25.2

[1] Rd values decrease as blackening increases. Each value is the mean of 2 determinations on 8 potatoes.

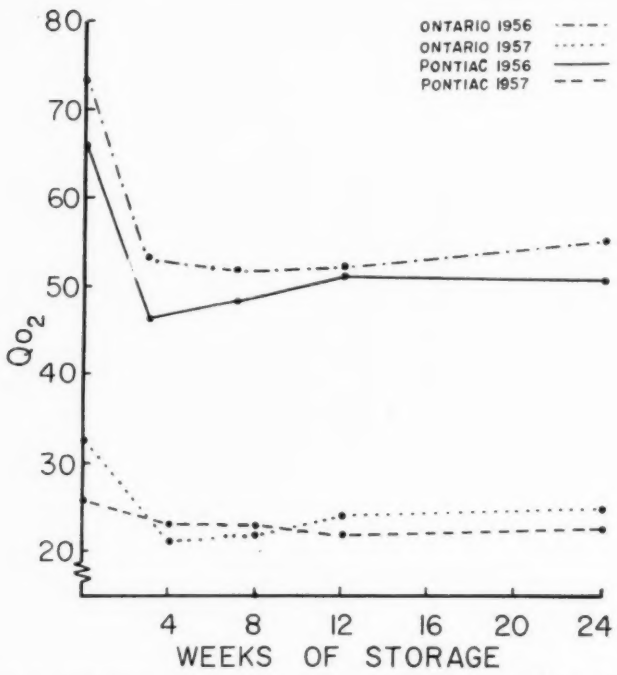

Figure 6. The effect of storage on the polyphenol oxidase activity of Pontiac and Ontario potatoes.

(Q_{O_2}: microliters of oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.) In 1956 the system included .2 ml potato extract and .2 ml catechol and in 1957 .4 ml of potato extract and .4 ml catechol. In 1956 data from 6 harvests are combined and in 1957 data from 3 harvests. Each value plotted is the mean of 18 determinations on 54 potatoes in 1956 and 6 determinations on 18 potatoes in 1957.

TABLE 2

Effect of maturity on potato discoloration after storage

Harvest	Weeks after planting	Variety	
		Pontiac	Ontario
1956		Rd [1]	Rd
1	7	.....	
2	9	32.0	37.3
3	11	30.2	31.3
4	13	33.0	29.5
5	15	31.3	28.9
6	17	31.8	25.6
1957			
1	10	31.1	26.2
2	13	28.3	22.1
3	17	33.5	23.3

[1] Rd values decrease as blackening increases. Each value is the mean of 10 determinations on 40 potatoes.

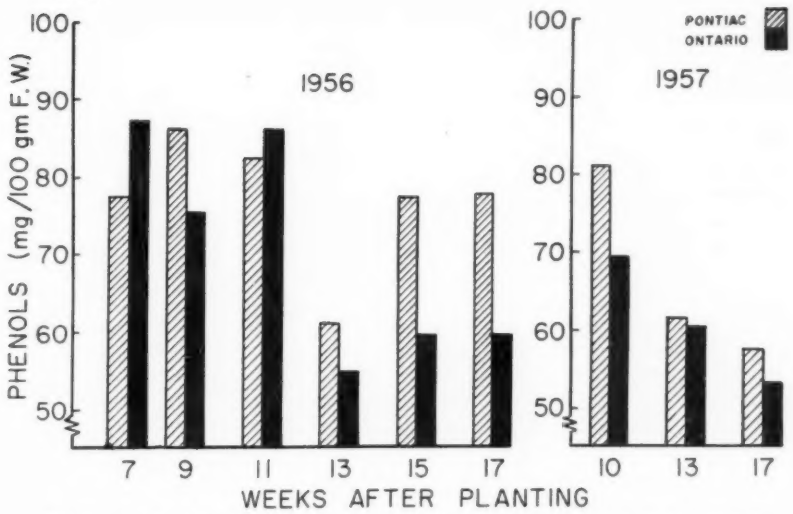

Figure 7. The effect of maturity on phenolic content of potatoes before storage. Each value plotted is the mean of 3 determinations on 12 potatoes in 1956 and 2 determinations on 8 potatoes in 1957.

small amount of polyphenol oxidase activity, were found to have the highest phenol concentration. It appears, therefore, that phenolic substances accumulate when the polyphenol oxidase activity is low and decrease in concentration as the enzyme activity increases.

Cytochrome oxidase activity appeared to be directly related to the concentration of phenolic substances in the potato. Immature potatoes, which were higher than mature potatoes in cytochrome oxidase activity, also had a higher concentration of phenolic substances. Both cytochrome oxidase

TABLE 3

Effect of storage on potato discoloration

Weeks of storage	Variety	
	Pontiac	Ontario
1956	Rd [1]	Rd
0	34.9	34.9
3	32.4	31.4
7	31.9	30.4
12	31.6	28.2
24	27.5	27.6
1957		
0	35.9	28.3
4	31.7	23.4
8	29.5	22.6
12	30.2	22.6
24	27.4	22.6

[1] Rd values decrease as blackening increases. Each value is the mean of 10 determinations on 40 potatoes in 1956 and 6 determinations on 24 potatoes in 1957.

activity and concentration of phenolic substances increased during the first three months of storage and was accompanied by an increased tendency toward discoloration. Joslyn and Ponting *(11)* suggested that cytochrome oxidase reacts with certain phenols and converts them into pigmented compounds similar to those observed in oxidations by polyphenol oxidase. The accumulation of phenols, and the increase in cytochrome oxidase activity in potatoes during storage, may account in part for the increased tendency of stored potatoes to darken.

One might expect that immature potatoes, which are higher in cytochrome oxidase activity and phenolic substances, would have a greater tendency toward discoloration than mature potatoes, but this relationship was not found. Phenolic substances may function quite differently in the young, rapidly-growing potato than that in the mature potato that has been stored.

After potatoes were stored polyphenol oxidase activity decreased at the same time that phenolic substances increased. Phenolic substances also may react with other compounds in the potato, or may serve as substrates for other enzyme systems which may be involved in potato discoloration. The Ontario variety, more susceptible to discoloration than the Pontiac variety, showed a much greater increase in phenolic content during storage.

The general findings in this study would indicate that discoloration may result from an accumulation of phenolic substances resulting from decreased

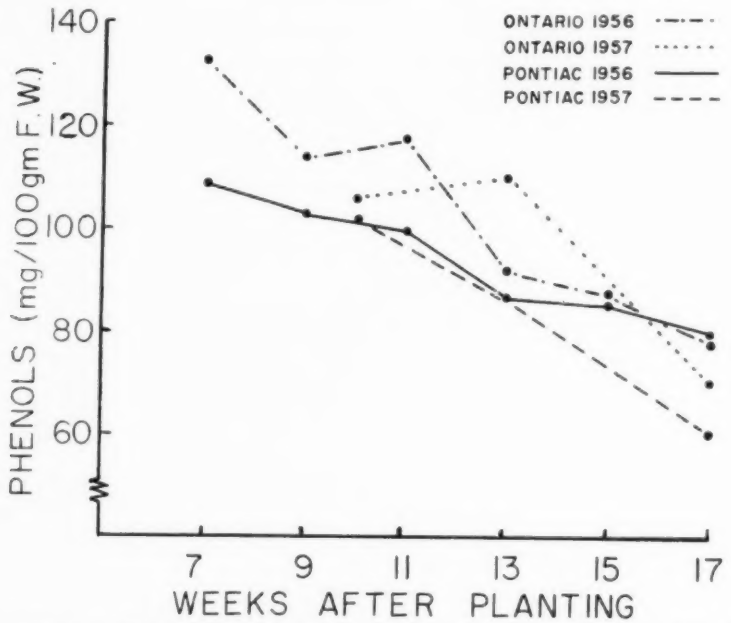

Figure 8. The effect of maturity and variety on phenolic content of potatoes after storage. Data from the 5 storage periods are combined. Each value plotted is the mean of 15 determinations on 60 potatoes in 1956 and 10 determinations on 40 potatoes in 1957.

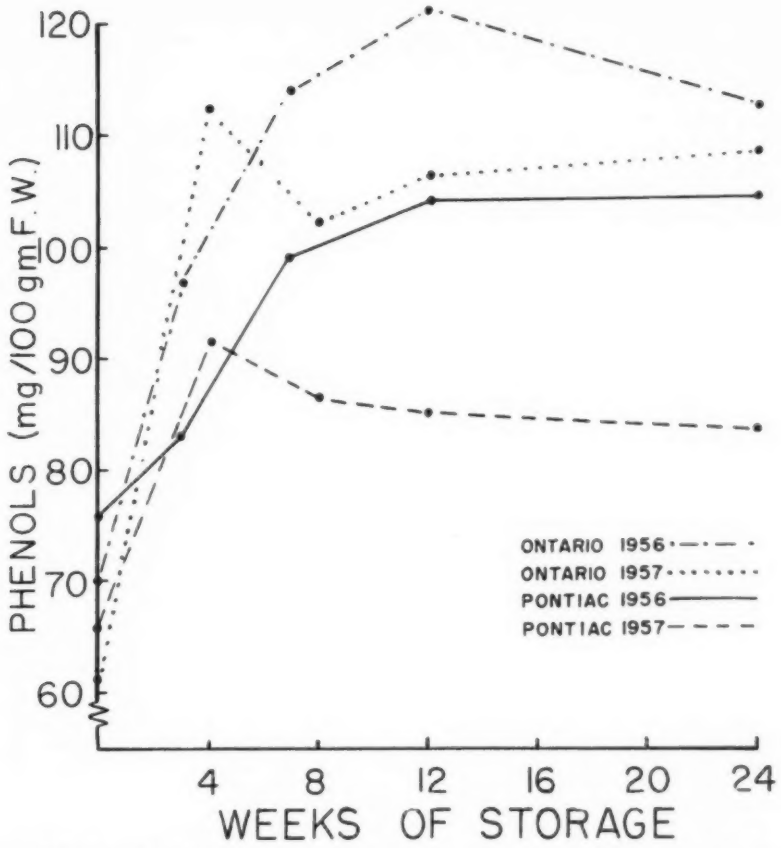

Figure 9. The effect of storage on the phenolic content of potatoes. In 1956 data from 6 harvests are combined and in 1957 data from 3 harvests. Each value plotted is the mean of 18 determinations on 72 potatoes in 1956 and 6 determinations on 24 potatoes in 1957.

activity of polyphenol oxidase. Enzyme systems other than polyphenol oxidase may be responsible for the formation of the dark pigment in potatoes following storage.

The sources and functions of phenols in the potato are not clear, nor is the role of polyphenol oxidase in the metabolism of the tuber clearly defined. More work is needed before the interrelationships of discoloration, phenolic content, cytochrome oxidase, and polyphenol oxidase activities of the tuber can be elucidated.

SUMMARY

Ontario and Pontiac potatoes, representing varieties susceptible and resistant to precooking blackening, were examined for discoloration, phenolic content, and cytochrome oxidase and polyphenol oxidase activities. Phenolic

content was measured by the method of Rosenblatt and Peluso, and cytochrome oxidase and polyphenol oxidase activities were determined by oxygen-uptake measurements. The Hunter Color Difference Meter was used to measure discoloration.

Studies were conducted for two consecutive years, 1956 and 1957. In each year the two varieties were grown under similar conditions and harvested at various stages of maturity. Determinations were made at the time of harvest, and after storage at 40° F for various periods of time through 6 months. Differences in discoloration, phenolic content, polyphenol oxidase and cytochrome oxidase activities due to storage, maturity and variety were highly significant.

The Ontario variety was found to be higher in phenolic content, cytochrome oxidase, and polyphenol oxidase activities than the Pontiac variety. Immature potatoes were found to be higher in phenolic content and cytochrome oxidase activity than mature potatoes. Discoloration was greatest in the most mature potatoes.

Cytochrome oxidase activity and the concentration of phenolic substances increased in tubers from the time of harvest up to three months of storage. Polyphenol oxidase activity decreased markedly during the first few weeks of storage and remained relatively low thereafter. As the polyphenol oxidase activity decreased, phenolic substances accumulated.

Discoloration of potatoes increased with storage duration and was accompanied by an increase in phenolic content and cytochrome oxidase activity and a decrease in polyphenol oxidase activity. Polyphenol oxidase activity appears to be inversely related to potato discoloration.

Acknowledgment

The authors wish to acknowledge the assistance of Drs. Ora Smith, Richard Sawyer, Robert Kunkel, Paul Muneta and Mr. George Collins, Department of Vegetable Crops, Cornell University, for the arrangements made in growing and harvesting the potatoes. Grateful acknowledgment is also made to Dr. John Skory for his guidance and assistance in the statistical analyses, and to Mmes. D. Boggs, M. McConnel, and H. Aller for their assistance in the laboratory analyses.

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THE ROLE OF FAT IN THE FLAVOR OF LAMB AND MUTTON AS TESTED WITH BROTHS AND WITH DEPOT FATS[a]

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(Manuscript received November 3, 1959)

Knowledge of the flavor of meat is limited, especially with regard to the flavor of lamb and mutton. Howe and Barbella *(3)* pointed out the complex nature of meat flavor. Crocker *(2)* indicated that the flavor of meat is derived from the hydrolysis of proteins, and is not associated with fat.

Pippen *et al (6)* showed that varying the amounts of fat in broths did not affect the intensity of chicken flavor. Extraction of fat did not change the flavor of chicken broths in experiments by Peterson *(5)*. Bouthilet *(1)* suggested that though extracted fat had no chicken flavor of its own it might absorb and carry flavor. In work with beef, Kramlich and Pearson *(4)* found that intensity of flavor and the fat content were not related.

Since the influence of fat on the flavor of lamb and mutton is not known, the present study was made on broths and depot fat of this species.

EXPERIMENTAL METHODS

Tests of mutton broths containing varying amounts of fat. Broths were prepared from ground meat of a ten-year-old Southdown ewe. The meat was lyophilized under a vacuum of 25–50 microns for 8–10 hr, and ground to a powder by use of a Wiley Laboratory Mill. In preparing the broths, powder and distilled water were combined in a glass container and heated over gently boiling water for 3 hr. A tightly-fitted lid allowed steam to recondense into the broth. The hot broth was filtered through glass wool, refiltered through Whatman filter paper (No 40), and put through a hand-homogenizer.

Fat-rich broths. The fat-rich broths were made after the meat had been lyophilized and ground to a powder. The fat inherent in the meat was retained. The proportion of meat powder to water was varied from 1 part meat with 2 parts water, to 2½ parts meat with 1 part water.

Fat-poor broths. For fat-poor broths, the same method of broth preparation was followed except that the lyophilized meat was pressed to remove a portion of the fat (Carver Press, for 8 min, at 8000 lb) before it was ground. The concentration of the broth was, in each trial, the same as the fat-rich broth with which it was compared.

Fat-free broths. After lyophilization and pressing, the residual fat was extracted from the meat. In Trial 2A, most of the fat was removed by washing with pentane, followed by heating over a steam-bath. Comments of the judges indicated that the pentane had not been completely removed and that fat extraction was incomplete. In subsequent trials, the sample was given a more extended extraction with pentane[d] for 7 hr in a Soxhlet apparatus and the solvent removed under vacuum (10 lb, 8 hr). These

[a] Scientific Paper No. 1906. Washington Agricultural Experiment Station, Pullman. Project 1375.

[b] The data are from a thesis presented to the Graduate School by Joyce Brown Hofstrand in partial fulfillment of the requirements for the degree Master of Science. Present address: Home Economics Experiment Station, University of Idaho, Moscow, Idaho.

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[d] Pure grade pentane, bp 36.5° C, Phillips Petroleum Co.

added precautions increased extraction and removed any evidence of pentane in the extracted powder or broth. The proportion of meat powder to water was varied from 2½ parts meat with 1 part water, to 1 part meat with 1 part water.

Organoleptic tests. Organoleptic tests were made by paired-comparisons. Ten experienced panel members compared the aroma and taste of the broths. The order of the samples was randomized and duplicate samples were included in each series. Warmed 40-ml samples were placed in 100 ml beakers. These beakers were without lip, and covered with watch-glasses. The beakers were enclosed in a styrofoam holder, Figure 1, to keep the samples warm during tasting. These holders, specially designed

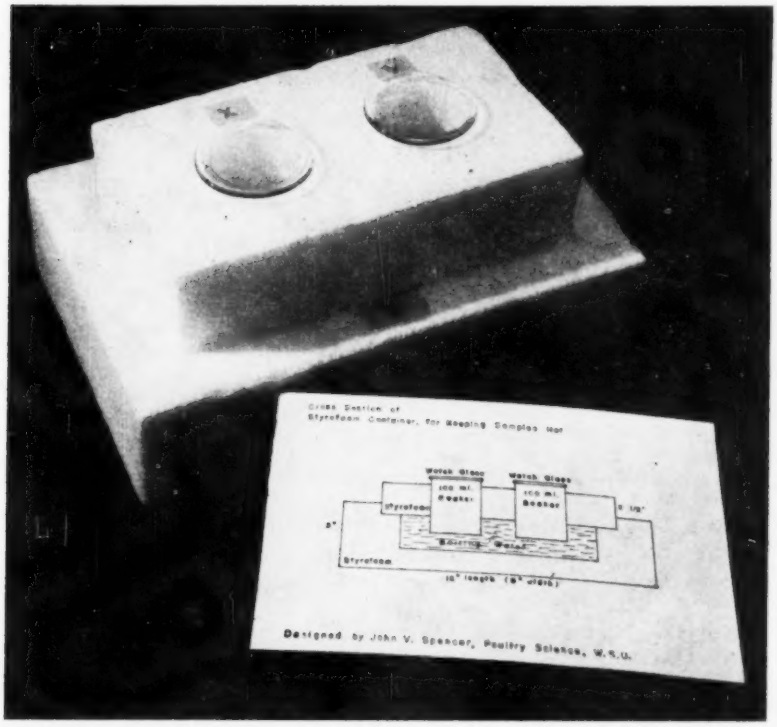

Figure 1. Styrofoam container for comparison of aroma and taste of warm samples.

for this study, proved to be ideal in maintaining temperature for several hours, and were non-absorbent and odorless.

In one series of comparisons (Trial 1C), a reference sample of cooked, ground lamb wrapped in foil was provided to observe the effect of such a reference on the accuracy of the identification of lamb flavor.

In another series of comparisons (Trial 2D), the unextracted meat was kept under the same conditions of heating as the extracted meat that had been in the Soxhlet for seven hours.

Tastings done individually were begun with Trial 2C and continued in that manner to the end of the experiment. Judges were taken one at a time into a well-ventilated, darkened room. The samples were presented as paired comparisons, and warmed in the styrofoam containers. Each judge's comments were noted while he was tasting, and if his description of a difference was not clear, he was questioned as to its meaning.

When such terms as "stronger," "watery," "meaty," "lamby," were used, a request for definition was made.

Decisions concerning the similarity or difference between the aromas of two samples, varying in amount of fat, were made by sniffing. In the later trials, judges were also asked to check descriptive terms ("lamb-like," sweet, sour, rancid, musty, "off," oily, "barny," or other) to characterize differences in aromas. For tasting, 5-inch lengths of quarter-inch glass tubing were used. Tasters measured an amount of sample up to a mark 1-inch from the end of the tubing. A clean tube was used for each sample.

Tests with Depot Fat

Depot fat from a yearling Southdown wether was pressed (Carver Press, 8 min, 8000 lb, 49° C). The expressed fat was collected, and the effects of the following treatments tested.

Heating expressed fat. Some samples of expressed fat were heated for 1 minute over direct heat. In a second test, other samples were given a milder heat treatment; for this, the fat was held over hot water (68° C) for 4 min. The treated fats were compared in aroma and taste with untreated fat.

Washing expressed fat with water. Sixty g. of expressed fat was washed with 180 ml of distilled water at 78° C. The water was mixed into the fat and allowed to remain in contact with the fat overnight, under refrigeration. Water drained from the fat was compared with distilled water. Washed and unwashed fats were also compared for aroma and taste.

Filtering through activated carbon. Expressed fat was treated with activated carbon to determine whether flavor components could be removed. Activated carbon (25 g) and expressed depot fat (80 g) were warmed together and filtered warm through Whatman filter paper (No 40). The filtration required about three hours at a temperature of 100° C. The aroma and taste of the carbon treated fat was compared with the untreated, expressed fat.

The procedures for comparing the aroma and taste of fat were similar to those used for broths. For tasting, however, a drop of fat from each beaker was sufficient. Also, soda crackers were used between samples to remove greasiness from the mouth.

Estimates of statistical significance. The statistical significance of the number of correct identifications of broths or fats prepared differently was estimated by the use of the chi-square.

RESULTS AND DISCUSSION

Flavor of fat-rich and fat-poor broths. When fat-rich and fat-poor broths were compared, the results were as shown in Table 1 (Trials 1A, 1B, 1C).

It appeared in the two replications of Trial 1A that the amount of fat may affect aroma but not taste. This is suggested by a recognition of difference in 50 of the 76 comparisons of broths prepared from pressed meat and unpressed control broths. Based on taste, there were no significant differences. Though the strengths of the broths had been increased in 1B and 1C, and a reference of cooked lamb given in 1C, differences were not found between the samples any more frequently than could be attributed to chance.

It appears that in fat-rich and fat-poor broths, the amount of fat did not significantly affect the taste but may affect aroma.

Flavor of fat-rich and fat-free broths. The effects of extracting residual fat by use of solvents upon the flavor of mutton broths are also shown in Table 1. No difference was noted in aroma between the fat-rich and fat-free broths, except in Trial 2D. Difference in taste was not significant in the 4 trials. Increasing the concentration of broths (Trial 2B) did not aid in distinguishing between them. In fact, several judges commented that these broths were too strong in flavor for tasting. The added precaution in Trial 2D, of keeping the unextracted meat at the same temperature as the meat in

TABLE 1

Comparisons of samples of broth

Trial	Number of replications	Total number of comparisons	Number of broths checked as "different"	
			Aroma	Taste
Fat-rich vs fat-poor				
1A—unpressed and pressed meat powders 1 part per 2 parts water	2	76	50 [1]	36
1B—concentration increased to 1 part meat powder per 1part water	2	72	40	35
1C—concentration same as 1B; reference provided	2	68	34	32
Fat-rich vs fat-free				
2A—extracted with pentane; concentration 1 part meat powder per 1 part water	2	72	39	33
2B—concentration increased to 2½ parts meat powder per 1 part water	2	96	53	48
2C—individual tasting; concentration decreased to 1½ parts meat powder per 1 part water	1	56	33	30
2D—unextracted, also heated; concentration as in 2C	1	54	35 [1]	33 [1]

[1] Significant at 5% level; all others are not significant.

the Soxhlet apparatus, may have made it possible to differentiate the aromas in that trial.

Flavor of depot fat. The results of investigation of the possible contribution of constituents dissolved in the fat to flavor (Trials 3A, 3B, 3C, 3D) are given in Table 2.

TABLE 2

Effect of treatments of depot fat upon flavor

Trial	Number of replications	Total number of comparisons	Number of fats checked as "different"	
			Aroma	Taste
3A—effect of direct heat	1	74	57 [1]	53 [1]
3B—effect of heating over water	1	76	61 [1]	58 [1]
3C—washed fat	1	36	28 [1]	26 [1]
wash water	1	36	28 [1]	26 [1]
3D—filtered through carbon	1	76	57 [1]	40

[1] Significant at the 1 per cent level.

Heating over direct heat for as short a time as 1 min changed the flavor to a strong "browned-fat" aroma and taste. This flavor was not described as "lamby." It was easily distinguished from the slightly warmed depot fat as is shown by the highly significant selections in the paired-comparisons (Table 2). Some of the typical lamb aroma must be easily driven off by heat. When the experiment was repeated with indirect heat (Trial 3B), that which was heated for 4 min was easily distinguished from the unheated fat. Even mild heating developed a meaty, fatty flavor which had lost most of its "lambiness." The judges commented that the heated fat could have been pork, beef, or other meat fat.

The results of Trial 3C indicated that one washing with water at 78° C for 10 min, and storage of the fat with the water under refrigeration for 16 hr

measurably changed the flavor of the fat. The washing water was easily distinguished from plain distilled water, indicating that soluble flavor components are present in depot fat of lamb.

When pressed depot fat was filtered through activated carbon (Trial 3D), the warm, filtered fat had the same kind of aroma as the unfiltered fat, but it was much less pronounced. There was very little taste to the filtered fat. The judges had no difficulty in distinguishing filtered and unfiltered fats by aroma, but they could not distinguish them by taste.

The findings in the trials with broths are similar to those of Crocker, Bouthilet, Pippen and Peterson using chicken broths, and Kramlich and Pearson in their work with beef. As with chicken and beef, the taste of lamb broth as prepared in this experiment was not affected significantly by the amount or absence of fat. The broths were distinguished slightly better by aromas than by tastes, but the differences, except in one instance, were not statistically significant.

Descriptive terms applied to the broths indicated that there may be a difference measurable by other techniques. In comments during the first tests, the broths with fat were repeatedly described as "lamby," "barny," and "animal-like." In succeeding tests, when a list of terms was checked, certain differences in aroma and taste became apparent from the comments. The numerical summary of the number of times these descriptive terms were checked is in Table 3.

TABLE 3

Descriptive terms for aroma and taste of fat-rich and fat-free broths

Terms	Aroma		Taste	
	Fat-rich	Fat-free	Fat-rich	Fat-free
Lamb-like	42	16	16	10
Sweet	36	0	42	0
Sour	0	7	0	3
Oily	16	0	47	0
"Barny"	27	3	13	4
Watery	3	1	28	1
Salty	0	6	0	32
Meaty	16	58	39	74
"Off"	4	11[1]	3	8[1]
Sharp	0	9	0	19
Mild	24	0	43	0

[1] In Trial 2A the pentane used as a solvent may not have been completely removed during drying and broth preparation.

This summary of descriptive terms, checked for aroma and taste, suggests that at least some of the judges found the fat-rich broths more "lamb-like," sweet, oily, "barny," and surprisingly, more mild than the fat-free. The descriptive terms also suggest that the fat-free broths were more sour, salty, "meaty," and sharp. Since such descriptions are highly subjective, they are recorded only to give direction to further research. Paired-comparisons did not show that the differences were significant. It may be that other techniques of preparation of sample or of panel testing could make it possible to measure more accurately the small differences in flavor of broths.

From the results of treatments used on depot fat, it appears that there are water soluble flavor components in depot fat that are very volatile and can be

driven off by heat. It may be that the characteristic "lambiness" is quite volatile. Samples kept warm for any length of time lost some of this characteristic. These experiments show that fat itself may not significantly alter the flavor of broths, but do not rule out the importance of volatile flavor components in the fat. Work with expressed fats gave more positive results than work with broths. Characteristic flavor evident in expressed fat may be lost during the manipulations used in broth preparation.

SUMMARY

A study was made on the role of fat in the flavor of lamb and mutton. Mutton broths of varying fat content, and treated samples of lamb depot fat were submitted to a taste panel as a series of paired-comparisons. From the differences found in the comparisons of broths, it was concluded that the amount or absence of fat did not significantly affect the taste of the broths. There was an indication, however, that fat may contribute to the aroma of lamb and mutton broths. When expressed fat was compared with the treated fats, depot fats were found to have flavor components. These flavor components in the fat were easily volatilized and changed by heat, soluble in cold water, and partially removed by activated carbon.

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SEPARATION AND IDENTIFICATION OF COOKED BEEF FLAVOR COMPONENTS[a,b]

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(Manuscript received December 12, 1959)

Although few would question the distinctive and desirable nature of cooked beef flavor, investigations to elucidate the responsible components have not been attempted until recent years. Probably the earliest attempt to study beef flavor was reported by Crocker *(2)* in 1948, who reported cooked beef flavor to be more odor than taste and indicated that hydrogen sulfide, amines of several kinds and possibly indole were present. Kramlich and Pearson *(3)* found that cooked and raw beef flavor appeared to reside largely in the water-soluble fraction. Burnett, Gehrke and Brady *(1)* isolated and identified acetaldehyde as the only volatile carbonyl compound present in vacuum dehydrated pork after storage at both −29° C and 34° C. Stahl *et al (5)* were able to separate by means of gas chromatography and identify with the aid of a mass spectrometer at least 38 different volatile compounds from raw beef after a beta radiation dosage of 4 megareps. However, only 5 compounds, carbon dioxide, carbon monoxide, hydrogen sulfide, methyl and ethyl mercaptan, were found to be present in the meat volatiles obtained from an unirradiated raw control sample. Merritt *et al (4)* also working with raw meat found acetaldehyde, acetone and methyl ethyl ketone to be present in both the unirradiated and irradiated samples. Trace amounts of methyl mercaptan, dimethyl sulfide, ethyl mercaptan, methanol and ethanol were identified. In addition, dimethyl sulfide and isobutyl mercaptan were found in irradiated raw meat.

Although considerable work has been done in identifying the volatiles in raw irradiated and unirradiated beef, the literature is devoid of information on the components responsible for cooked beef flavor. Consequently, this investigation was undertaken to separate and identify the volatile components from cooked beef, which may possibly contribute to its flavor.

EXPERIMENTAL

Fractionation and identification of volatile sulfide and carbonyl compounds. The beef was ground once through a quarter inch plate and then weighed. Sufficient de-ionized distilled water was added to make a 1-3 meat-water dilution by weight. The water and meat were blenderized and poured into a 12 liter, triple-necked, round bottom flask. The flask was connected to a series of traps for the isolation of volatile sulfides and carbonyls, which were similar to that described by Pippen *et al (5)* except nitrogen was used as a carrier gas instead of utilizing vacuum. The first trap contained lead acetate solution, while the second and third each contained 300 ml of a saturated 2,4-

[a] Journal Article 2546, Michigan Agricultural Experiment Station, East Lansing.
[b] This paper presents a portion of the research reported in the Ph.D. thesis submitted by the senior author to the School for Advanced Graduate Studies at Michigan State University.
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dinitrophenylhydrazine solution (2 g per liter in 2N HCl). In this system, the lead acetate trap removed the volatile sulfur-containing compounds, while the carbonyl compounds were removed by the 2,4-dinitrophenylhydrazine reagent by formation of their respective insoluble hydrazones.

Eight hundred grams of meat were cooked vigorously for 24 hr, after which the flask was emptied and then recharged with an additional 700 g of meat. Cooking was continued for another 96 hr. Nitrogen gas was continuously bubbled through the cooking slurry to convey the meat volatiles through the system of traps. At the end of the cooking period, the hydrazones collected in traps 2 and 3 were filtered off, washed with acidified water and dried under vacuum.

Identification of the hydrazones was attempted using ascending paper chromatography. The solvent used was a mixture of 95% ethyl ether and 5% petroleum ether.

Fractionation and identification of total cooked beef volatiles. A 1–3 meat-water slurry was prepared as described previously and poured into a 12-liter round bottom flask. The apparatus used for the fractionation of total cooked beef volatiles is shown in Figure 1. The first trap was immersed in a beaker of wet ice, the second in a beaker

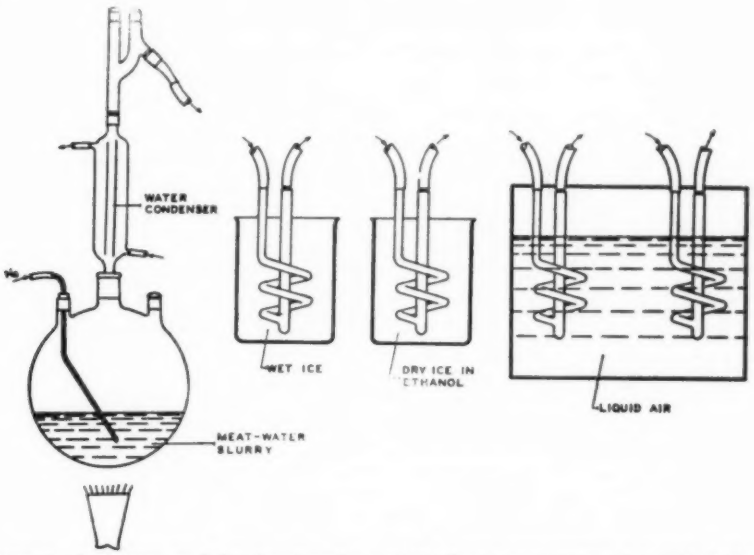

Figure 1. Apparatus used for fractionating volatile flavor components of cooked beef.

containing ethanol and dry ice. The third and fourth traps were immersed in liquid air with a block of styrofoam being utilized to contain the liquid-air. The meat slurry was cooked 5–7 hr with nitrogen gas constantly bubbling through it. After the cooking was completed, the traps were disconnected, stoppered, and placed aside for subsequent fractionation of the volatile components in a Perkin-Elmer, Model 154-B, vapor fractometer.

Although a number of columns was tried, identification of the compounds was attempted on only the three best columns—dinonylphthalate, didecylphthalate and "Carbowax 400"—which are reported in this study. The stationary phase was carried out on 30- to 60-mesh acid washed celite.

The trap containing the volatiles was removed from the liquid air or dry ice and ethanol and either immersed in hot Dow-Corning 550 oil or heated directly with the flame of a Bunsen burner to bring about faster volatilization. Helium was diverted into the trap for 15 seconds to flush the volatiles out of the trap and into the column. Identifica-

tion of the fractionated components was attempted by comparing the retention volume for each unknown volatile component with the retention volume of known compounds after resolution on the same column.

A second method of identification employed the use of specific qualitative tests for determination of various fractions. Each of these tests involved the bubbling of the gas responsible for a particular peak through the test solution as the gas emerged from the column.

A third attempted method of identification involved infrared spectrophotometry. A double-beam infrared spectrophotometer equipped with a salt prism was used for analysis of the total volatile mixture.

RESULTS

Identification of volatile sulfide and carbonyl compounds. The presence of a black precipitate in the lead acetate trap was observed, this being indicative of the presence of volatile sulfide compounds. However, no attempt was made to separate and identify the sulfur-containing compounds in the precipitate.

Figure 2 shows a paper chromatogram of the carbonyl compounds chromatographed as their hydrazone derivatives. Acetaldehyde was identified as being one of the spots, but the other two spots showed evidence of excessive trailing, making identification impractical.

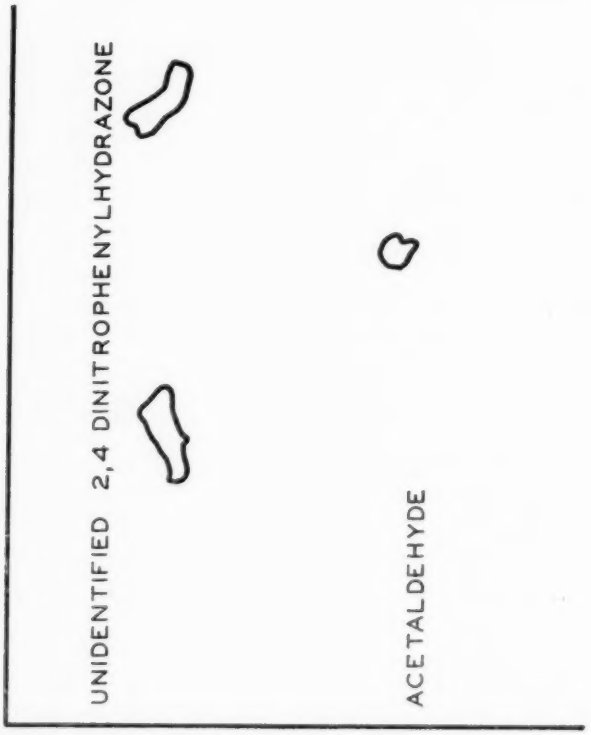

Figure 2. Paper chromatogram showing carbonyl compounds as hydrazone derivatives. Solvent flow—6 hours (ascending) solvent system—95% petroleum ether—5% ethyl ether.

Identification of total cooked beef volatiles. The major share of identification of the volatile compounds fractionated in the various cold traps, and then further fractionated in a vapor fractometer, was accomplished by comparing retention volumes from gas chromatograms. Retention volumes were calculated from a standard calibration curve for both known compounds and the fractionated volatiles. Due to the nature of the curve, all retention volumes based on the curve are considered to be approximations and not exact values. Of the three different columns used, both dinonylphthalate and didecylphthalate resolved the volatiles collected in the liquid air trap into 6 peaks, while the "Carbowax 400" column only resolved the mixture into 3 peaks.

Didecylphthalate column. The trap was removed from the liquid air and immersed in warm oil (approximately 45° C) for 10 min prior to injection into the vapor fractometer. Six peaks were shown on the chromatogram. The comparative retention volumes of the unknown and the known compounds resolved by the didecylphthalate column are shown in Table 1. Peak one was identified as carbon dioxide by bubbling

TABLE 1

Retention volumes of known and unknown compounds resolved on a didecylphthalate column

Unknown compounds [1]			Known compounds [1]	
Fig No	Peak No	Retention volume (cc He)	Compound	Retention volume (cc He)
3	1	70		
	2	99		
	3	120	Methyl mercaptan	112
	4	167	Acetaldehyde	152
	5	187		
	6	220	Acetone	227

[1] The known compound on the same line with an unknown compound indicates tentative identification of the unknown on the basis of similar retention volumes. Blank spaces in the column for known compounds indicate that identification was not possible.

the gas emerging from the vapor fractometer through a saturated calcium oxide solution and observing the white precipitate formed. Attempts to obtain a retention volume for a known carbon dioxide sample were unsatisfactory.

Peak two was not identified. Its lack of size and rounded contour did not lend itself to identification by comparison with known compounds, nor did the small quantity of gas present allow for identification by means of various qualitative tests. The third peak had a retention volume of 120 which is similar to that for methyl mercaptan. A yellow precipitate was observed when the gas was bubbled through a saturated solution of lead acetate in ethanol, which tended to verify the identification.

The retention volume of peak four on the didecylphthalate column was 167, which is approximately that of acetaldehyde. Upon bubbling the gas through a solution of 2,4-dinitrophenylhydrazine, hydrazones were precipitated, which indicated the peak to be a carbonyl compound. Due to the lack of good separation and resolution, peaks five and six could not be identified.

"Carbowax 400" column. The "Carbowax 400" column resolved only 3 peaks compared to the 6 peaks resolved by the phthalate columns. Retention volumes for all known and unknown compounds resolved on the "Carbowax 400" column are shown in Table 2. Peak 1 was identified as carbon dioxide by observing a white precipitate when the gas was bubbled through a calcium oxide solution. Peak 2 showed a retention volume similar to that of methyl mercaptan. The gas emerging from the vapor fractometer did not give a reaction to the isotin or nitroprusside tests for methyl mercaptan, but did give a slight yellow precipitate when bubbled through an ethanolic solution of lead acetate.

TABLE 2

Retention volumes of known and unknown volatile compounds resolved on "Carbowax 400" column

Unknown compounds [1]			Corresponding known compounds [1]	
Fig No	Peak No	Retention volume (cc He)	Compound	Retention volume (cc He)
4	1	198	.....	
	2	381	Methyl mercaptan	367
	3	611	Acetaldehyde	625

[1] The known compound on the same line with an unknown compound indicates tentative identification on the basis of similar retention volumes. Blank spaces in the column for known compounds indicate that identification was not possible.

The retention volume of peak 3 compared favorably with that of acetaldehyde. Hydrazones were formed when the gas was bubbled through 2,4-dinitrophenylhydrazine, indicating peak 3 to be a carbonyl compound and giving supporting evidence to its identification as acetaldehyde.

A slight but distinct odor emanated from the dry ice-ethanol trap after each collection was completed. This indicated that one or more volatile compounds were being collected in this trap and was not allowed to pass into the traps immersed in liquid air. A gas chromatogram of these volatiles showed only 2 peaks. The first peak was poorly resolved and could not be identified while the second was tentatively identified as water.

Dinonylphthalate column. This column resolved the volatile mixture of the compounds obtained upon heating the beef slurry into 6 peaks. Table 3 shows the

TABLE 3

Retention volumes of known and unknown volatile compounds resolved on a dinonylphthalate column

Unknown compounds [1]			Known compounds [1]	
Fig No	Peak No	Retention volume (cc He)	Compound	Retention volume (cc He)
5	1	68	.....	
	2	93	Methyl mercaptan	105
	3	172	Acetaldehyde	180
	4	225	Methyl sulfide	229
	5	298	Acetone	279
	6	304	Water	323

[1] The known compound on the same line as the unknown compound indicates tentative identification of the unknown on the basis of similar retention volumes. Blank spaces in the column for known compounds indicate identification was not possible.

comparative retention volumes of the unknown compounds as compared to the known compounds. Peak one was tentatively identified as carbon dioxide by passing the gas through calcium oxide and observing the precipitate. Peak two showed a retention volume approximating that of methyl mercaptan. A yellow precipitate was noted when the gas was bubbled through a solution of lead acetate in 95% ethanol. A retention volume, which closely paralleled that of acetaldehyde, was observed for the third peak. Bubbling the gas through 2,4-dinitrophenylhydrazine precipitated hydrazones, indicative of a carbonyl compound and thus further verified the identification.

Peak 4 was found to have a retention volume similar to that of methyl sulfide. However, bubbling the gas through a lead acetate solution failed to give a positive test for sulfides. Peak 5 produced a few hydrazone crystals when bubbled through 2,4-dinitrophenylhydrazine, indicating a probable carbonyl compound. The retention volume more closely approximated the retention volume of acetone than any other compound tested.

Peak 6 showed poor resolution at the end of 2 min but was resolved much more satisfactorily after a volatilization period of 10 min. A retention volume similar to that of water was noted.

Thus, carbon dioxide, methyl mercaptan and acetaldehyde were identified as being present in the volatile mixture, which agrees with the results obtained using the didecylphthalate and "Carbowax 400" columns. Acetone was also resolved, as it was on the didecylphthalate column. Identification from the gas chromatograms made with the dinonylphthalate column, coupled with quantitative tests on the peaks as they emerged from the column indicated that acetone, water and methyl sulfide were also present.

Infrared spectrophotometry. Infrared absorption maxima were noted at 4.3, 6.6 and 12.2 mc. However, no conclusions as to the identification of the various components could be drawn from the graph. Apparently, the concentration of the volatile components was too low to be accurately identified by this method.

DISCUSSION

Of the volatile carbonyl compounds collected and chromatographed on paper as 2,4-dinitrophenylhydrazone derivatives, only acetaldehyde was identified; however, the presence of at least two, and possibly more carbonyls was indicated by the presence of two large, poorly resolved spots.

The presence of sulfur compounds in the mixture was indicated by passing the volatiles through a lead acetate solution and observing the black precipitate formed. Identification by means of gas chromatography is schematically summarized in Figure 3. The 3 peaks resolved by the "Carbowax 400" column were identified as were all 6 peaks resolved by the dinolylphthalate column. However, only three of the 6 peaks resolved by the didecylphthalate column were identified. Carbon dioxide, methyl mercaptan and acetaldehyde were identified by qualitative tests or retention volumes, or both, with all 3 columns. In addition, acetone was identified with both the phthalate columns and methyl sulfide and water were tentatively identified with the dinonylphthalate column.

An infrared spectrum of the entire volatile mixture showed but three relatively small absorption maxima. No conclusions as to the identification of the various components could be drawn from the graphs.

The components fractionated and identified in this study are believed to contribute to the flavor of cooked beef. The results obtained using paper chromatography, plus the fact that on occasions more than 6 peaks were obtained using the phthalate columns in the vapor fractometer would seem to indicate that more compounds are involved in the flavor of cooked beef than were detected during this investigation. Such additional compounds even though present in small quantities, could conceivably contribute greatly to beef flavor.

Bubbling of the mixed volatiles through distilled water as they emerged from the heated meat-water slurry produced a somewhat beefy flavor. This indicated that the volatiles in combination appeared to account for at least part of the characteristic flavor of beef. However, the relative importance of the different components identified cannot be assessed on the basis of the information obtained in this study. Resolution of their contribution to beef flavor can only be accomplished by testing them singly and in combination with a reference standard of cooked beef flavor. Furthermore, it is quite likely that all compounds contributing to beef flavor are not volatile, and thus the problem of duplicating flavors would become more difficult.

In conventional cooking of meat in air, some of the volatile products may

be the result of oxidation and could conceivably contribute to meat flavor, whereas, in this study oxidation was minimized by heating under an inert atmosphere of nitrogen. It is recognized that the volatiles collected in this study were obtained on boiling meat, while other methods of cookery, such as broiling, roasting or frying involve surface charring and oxidation and could conceivably yield different volatile components. On the other hand, oxidation of the internal portion of any meat product during cooking in air is probably negligible. Furthermore, the meat-like flavor obtained on bubbling the volatile mixture through distilled water would indicate that the major flavor components are not a result of such oxidation. The possibility never-

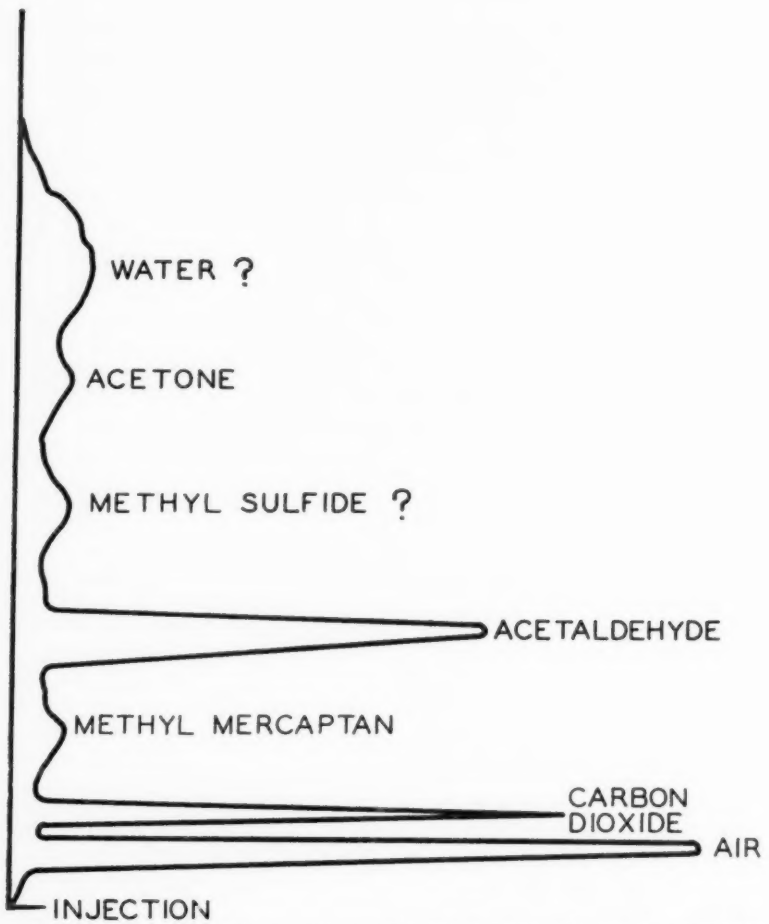

Figure 3. Composite gas chromatogram showing schematic diagram of compounds identified on all columns.

theless exists that a number of components of meat flavor were absent from the volatile mixture or present in smaller amounts.

CONCLUSIONS

Volatiles were collected in a series of cold traps on heating a meat-water slurry and using nitrogen as a carrier gas. Use of paper and gas chromatograms coupled with qualitative tests indicated that carbon dioxide, methyl mercaptan, acetone and acetaldehyde were present in the volatile fraction from cooked beef. In addition, methyl sulfide and water were tentatively identified as being present. Although a somewhat beef-like flavor was produced on bubbling the mixed volatiles from cooked beef through distilled water, the relative importance of the different components identified as contributors to beef flavor remains to be resolved.

Acknowledgment

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QUANTITATIVE ASPECTS OF THE INTERACTION OF CARRAGEENAN WITH CATIONIC SUBSTANCES. I. INTERACTION WITH METHYLENE BLUE

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Carrageenan reacts with methylene blue to form a blue, fibrous precipitate which is insoluble in most organic solvents and even in boiling water. This is due to an interaction between the negatively charged mucilage and the positively charged dye. Ewart and Chapman *(2)* employed this behavior of the widely used food stabilizer to advance a scheme for its qualitative identification. Stoloff *(8, 9)* refers to the reaction as being highly specific for carrageenans or polysaccharides related to Irish moss extractive. Jacobs *(5)* and the *Official Methods of Analysis (7)* recommend it as the confirmatory test for this food additive. As early as 1930, Ewe *(3)* reported on the distinct nature of these "blue insoluble clots" and suggested that the reaction could be used for the identification and differentiation of carrageenan from other plant gums and probably for its quantitative estimation. However, as far as is known, no detailed study of the quantitative nature of this interaction has been made.

Since this highly insoluble precipitate is so unique for carrageenan and polysaccharides related to carrageenan, the possibility of using it for characterization and quantitative estimation looms, and should aid in any final scheme for the separation, identification, and quantitative determination of food stabilizers. Such a test could serve as a measure of the polysaccharide(s) in fundamental studies on reactions with specific chemical and biochemical entities.

The studies reported herein were designed to explore the quantitative nature of the interaction of methylene blue with carrageenan. The factors which influence the interaction were investigated and conditions for quantitative relationships established.

EXPERIMENTAL METHODS AND RESULTS

Reagents:[a]

Seakem Type 3: Batch No. 262808, moisture content—12.05%
Seakem Type 5: Batch No. 252407, moisture content—10.9 %
Seakem Type 7: ————————, moisture content—11.4 %
Seakem Type 402: Batch No. 190218, moisture content—11.95%

Stock solutions of 0.5% (dry weight basis) were prepared by dusting weighed amounts of the dried commercial products onto distilled water heated to 70° C. Constant stirring was done with a glass rod. After cooling, the mixtures were transferred to volumetric flasks and made up to volume with distilled water. When necessary, appropriate dilutions were made with distilled water.

Methylene Blue N.F. (powder-zinc free):[b]

[a] All supplied by Marine Colloids, Inc., New York, New York, and New Bedford, Massachusetts.

[b] Supplied by The Carolina Biological Supply Company, Elon College, North Carolina, 98.5% purity on a moisture free basis.

Purity of the product was checked according to the method of *(7)*. Stock solutions at 1400 μM/L were made up daily by placing 0.5 g of the dried (vacuum desiccator) commercial product in a 100 ml volumetric flask, dissolving, and making up to volume with distilled water.

1M H_3PO_4: 62.8 ml of commercial orthophosphoric acid (85% H_3PO_4 specific gravity 1.834) were placed in a volumetric flask and diluted to 1000 ml with distilled water. When necessary, appropriate dilutions were made with distilled water.

0.1 M $(NH_4)_2HPO_4$: 13.211 g of the A.C.S. grade chemical were placed in a volumetric flask and diluted to 1000 ml with distilled water.

0.1 M NH_4OH: C.P. Ammonium Hydroxide (28% NH_3, specific gravity 0.9015) was used. 13.75 ml of the commercial product were placed in a volumetric flask and made up to 1000 ml with distilled water.

Acidified absolute ethyl alcohol and absolute methyl alcohol: Analyzed reagent grade chemicals were used. One ml of 1 M H_3PO_4 was added per 100 ml of alcohol.

0.2 N iodine solution: This was prepared according to Kolthoff and Sandell *(6)*.

0.1 N sodium thiosulfate solution: This was prepared and standardized according to *(6)*.

0.2% starch solution:

Double distilled water: This was twice distilled from glass.

Equipment:

1. Volumetric pipettes
2. Coleman Universal Spectrophotometer, Model 14
3. Large borosilicate test tubes of 120 ml capacity
4. Hard rubber stoppers
5. 30° C incubator
6. 50 ml burette
7. Glass stoppered pyrex test tubes of 25 ml capacity

GENERAL PROCEDURE AND ESTABLISHMENT OF STANDARD CURVE

Fourteen ml of the acidified ethyl alcohol were added to each of 7 glass stoppered pyrex test tubes. Methylene blue was added to consecutive test tubes to make up solutions containing 0 to 8.4 μM/L. The total volume of each tube was made up to 20 ml with distilled water, where necessary. The final alcohol concentration in each tube, therefore, was essentially 70%. The tubes were well shaken and the intensity of the color measured with a Coleman Model 14 Spectrophotometer at 650 mmc with acidified alcohol as the blank.

The appropriate wave length of maximum absorption was determined from a plot of the absorbency as a function of the wave length as shown in Figure 1. A plot of the logarithm of the per cent transmission as a function of the concentration of methylene blue gave a standard linear curve indicating that Beer's law was being obeyed. Under the experimental conditions employed, the relationship may be expressed by the following logarithmic least squares equation:

$$X = \frac{2.3944 - \log \% T}{0.158}$$

where X = concentration of methylene blue in micromoles per liter.

Exactly the same procedure was used for studying the uptake of methylene blue by carrageenin. Mixtures of the dye and gum were allowed to come to equilibrium in 0.05M $(NH_4)_2HPO_4$—NH_4OH buffer of pH 9.0. An aliquot was pipetted off and centrifuged at 2500 rpm for 10 min to sediment the precipitate. One ml of the clear supernatant was diluted 1:10 with distilled water. Two ml of this diluted sample were added to 14 ml of the acidified alcohol and the total volume made up to 20 ml with distilled water. The contents of the tube were mixed well and the color intensity measured as described above. The quantity of methylene blue present was determined either from the standard curve or from the least squares equation.

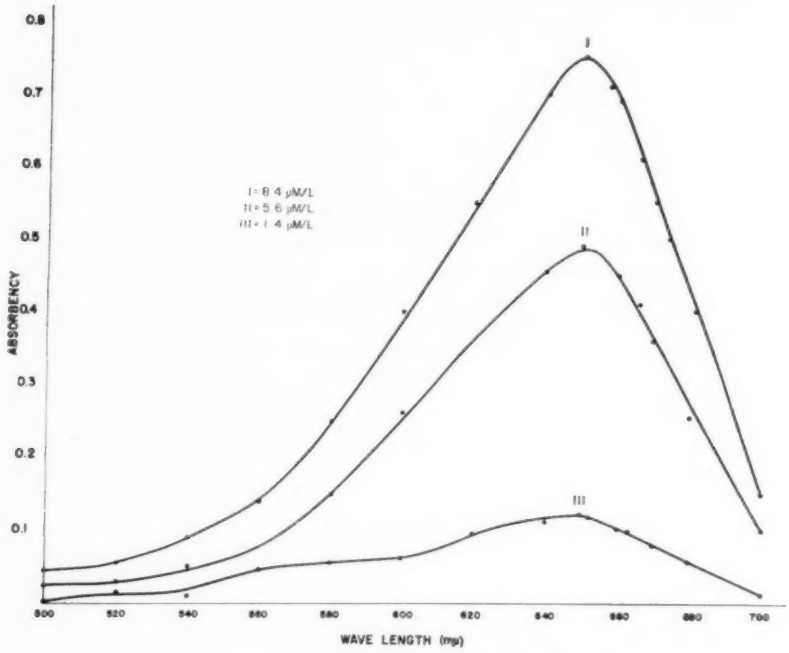

Figure 1. Absorption spectrum of methylene blue in 70% ethyl alcohol.

EFFECT OF VARIABLES ON THE COLOR INTENSITY OF METHYLENE BLUE

In order to determine the stability of methylene blue in acidified ethyl alcohol, duplicate solutions containing 5.6 μM/L of methylene blue were made up in acidified ethyl alcohol according to the general procedure. The color intensity of each solution was measured after time lapses of 0, 12, 24, 36 and 48 hr. Zero time was taken as 10 min after the preparation of the last tube of solution. This time lapse allowed for necessary manipulations. The results recorded in Table 1, indicate that the color of methylene blue in 70% acidified ethyl alcohol is stable for as long as 48 hr, the longest period tested.

There existed the possibility that if carrageenan, buffer, and methylene blue were all present in the system at the same time, some methylene blue—carrageenan complexes could be formed. Such complexes in the supernatant could cause a shift in the wave length of maximum absorption as compared to that of a solution of methylene blue and alcohol only. Therefore, this possibility was examined. Three mg of carrageenan Type 3, 5.6 μM of methylene blue, and 20 ml of the pH 9.0 buffer were mixed in large borosilicate test tubes and incubated in a 30° C water bath for 24 hr. After appropriate dilution, the absorption spectrum of each supernatant was determined between 500–700 mmc using a Beckman Spectrophotometer Model 14 and acidified ethyl alcohol as the blank. The results of duplicate determinations are summarized in Figure 2. Since the absorption spectrum of the dye in the

TABLE 1

Effect of variables on color intensity of methylene blue and on the dye-gum interaction

VARIABLE	Average % transmission at 650 mμ
Stability of methylene blue in acidified ETOH	
Time lapse (hrs)	
0	32.6
12	32.7
24	32.4
36	32.3
48	32.4
pH of reacting medium	**Methylene blue taken up per mg of Seakem Type 3**
	μM
3.5	1.70
4.6	2.01
6.4	2.20
7.8	2.44
8.8	2.43
9.8	2.44
Buffer strength (M)	
0.1	2.39
0.05	2.43
0.01	2.41
Equilibrium time	
5 min	1.22
10 min	1.50
15 min	1.70
30 min	2.11
45 min	2.20
60 min	2.31
90 min	2.37
2 hr	2.42
4 hr	2.43
8 hr	2.47
12 hr	2.42
Methylene blue concentration (μM/L)	
56.0	0.231
280.0	1.22
420.0	1.73
560.0	2.28
840.0	2.50
1120	2.49

supernatant showed the same maximum as methylene blue in acidified alcohol, it is evident that either no complexes were formed through interaction between the gum and the dye or, alternately, if such was the case, the complex decomposed when the supernatant was diluted with acidified ethyl alcohol.

To assess the influence of alcohol concentration on the wave length of maximum absorption of methylene blue solutions, duplicate tubes containing 5.6 μM/L of methylene blue and 0, 10, 20, 40 and 80% acidified ethyl alcohol, were prepared. Determinations were made as in previous experiments and the results summarized in Figure 2. At and above 10% alcohol concentration, the wave length of maximum absorption of methylene blue is at 650 mμ

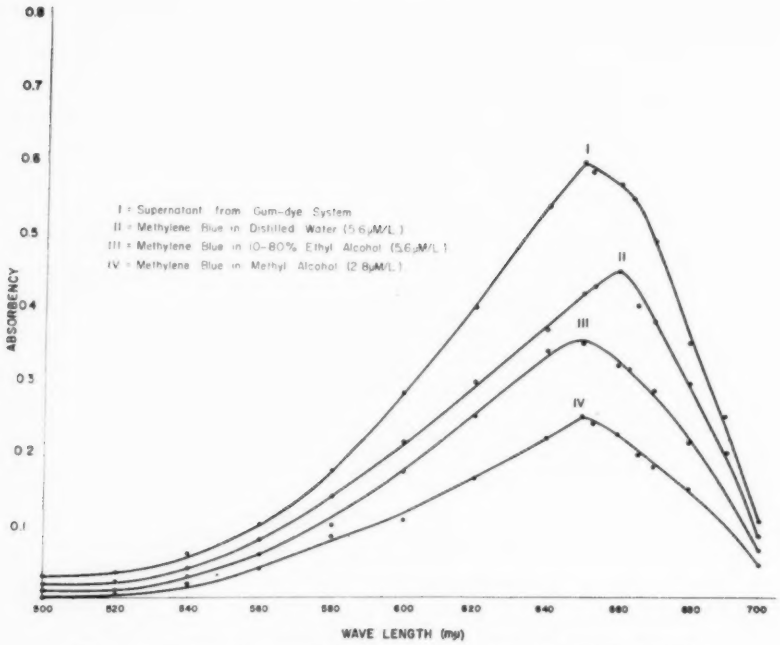

Figure 2. Absorption spectrum of methylene blue in various systems.

and the per cent transmission over the wave length range investigated is, within experimental error, the same. However, in distilled water the wave length of maximum absorption is at 660 mmc. This latter value agrees well with previous measurements of 660 mmc and 665 mmc reported by other workers *(1, 10)*.

Information as to whether ethyl alcohol and methyl alcohol were interchangeable was obtained by using duplicate solutions of 5.6 μM/L solutions of methylene blue in acidified 70% concentrations of both alcohols. It was found that any differences resulting when either alcohol was used was well within the range of experimental error.

INFLUENCE OF VARIABLES ON THE INTERACTION

The results of studies on the influence of several variables on the interaction between methylene blue and carrageenan type 3 are recorded in Table 1.

The time necessary for equilibrium to be achieved was determined by placing 10 mg of carrageenan type 3 and 42 μM of methylene blue in large borosilicate test tubes. Twenty-five ml of 0.1 M $(NH_4)_2HPO_4 - NH_4OH$ buffer of pH 9.0 were added and the total volume made up to 50 ml with distilled water. The tubes were incubated in a water bath at 30° C. At intervals indicated in Table 1, aliquots were taken from separate tubes, centrifuged for 10 min at 2500 rpm and the appropriately diluted supernatant used for assay of methylene blue according to the general procedure. The results

of duplicate determinations indicate that equilibrium was reached after incubation for 2 hr, and that there was no change after as long as 12 hr. For experimental convenience, all subsequent incubations were done for 12 hr at 30° C. However, where speed is essential such as in routine laboratory work, an equilibrium time of 2 hr is more convenient. Furthermore, any period between 90 min to 2 hr, if used consistently will lead to essentially the same or closely similar results.

In order to study the influence of pH, buffers of varying pH values were prepared by mixing 0.1 M $(NH_4)_2HPO_4$, 0.1 M NH_4OH and 0.1 M H_3PO_4. Carrageenan Type 3 and methylene blue were mixed and incubated in exactly the same way as in the study of equilibrium time and the uptake of methylene blue at each pH determined after the general procedure. The initial pH and the final pH of each mixture were checked using a Beckman Model G pH meter. As the pH increased, the amount of dye taken up increased. This suggests that the interaction is primarily electrostatic in nature. At the lowest pH, the value obtained could have been unreliable since there are reports of hydrolysis *(8)* at low pH levels. However, Hansen *(4)* could detect no decomposition at acidities as low as pH 3.0. Since buffer capacity is greatest at around pH 8.5 to 9.0, and the uptake between pH 7.8 and 9.8 is essentially the same, a pH of 9.0 was selected for all subsequent studies. In addition, the stability of carrageenan is, reportedly *(8)*, greatest at pH 9.0.

The concentration of methylene blue necessary for saturation of the anionic sites of the hydrocolloid at equilibrium was studied by mixing 10 mg of carrageenan type 3 and varying concentrations of methylene blue and incubating the mixture in the usual manner. The uptake of the dye at each added level was determined by the usual procedure. Under the experimental conditions employed, saturation of all available anionic sites occurs when each gram of the polysaccharide has associated with it 2.5 μM of methylene blue. Therefore, a solution of 840 μM/L of methylene blue (total volume 50 ml) was used in future studies for levels of 10 mg or less of carageenan.

For studying the influence of buffer molarity, buffers of molarities indicated in Table 1, all pre-adjusted to pH 9.0, and the procedure of previous experiments were used. The results indicate that buffer molarity is of primary importance only in the final stage. Since alcohol was used as the diluent for color measurement, molarities higher than 0.1 were impractical since excessive dilution would be necessary to avoid cloudiness. At molarities of 0.01 to 0.1, the uptake of methylene blue at pH 9.0 was found to be essentially the same. A final molarity of 0.05 was chosen.

UPTAKE OF METHYLENE BLUE BY INCREASING QUANTITIES OF CARRAGEENANS

Increasing quantities of the carrageenan samples were added to large borosilicate test tubes. Forty-two μM of methylene blue and 25 ml of 0.1 M $(NH_4)_2HPO_4 - NH_4OH$ buffer, pH 9.0, were added to each test tube. The total volume of each test tube was made up to 50 ml with distilled water. The tubes were incubated in a 30° C water bath for 12 hr. After this period, an aliquot was pipetted off and centrifuged at 2500 rpm. One ml of the clear supernatant was used for methylene blue assay as outlined in the general procedure.

In order to ascertain if any material(s) in the carrageenan was interfering, a portion of each sample was dialyzed for 30 hr in $^{36}/_{32}$ dialysis membrane against 6 changes of double distilled water at 5° C. The uptake of methylene blue was determined as before.

The results summarized in Table 2 and Figure 3 show that a plot of the amount of methylene blue taken up as a function of the amount of added carrageenan gives a straight line. This is indicative of a quantitative interaction between the dye and the gum. No important differences were found between the dialyzed and non-dialyzed samples indicating that either dialyzable materials did not interfere or were absent.

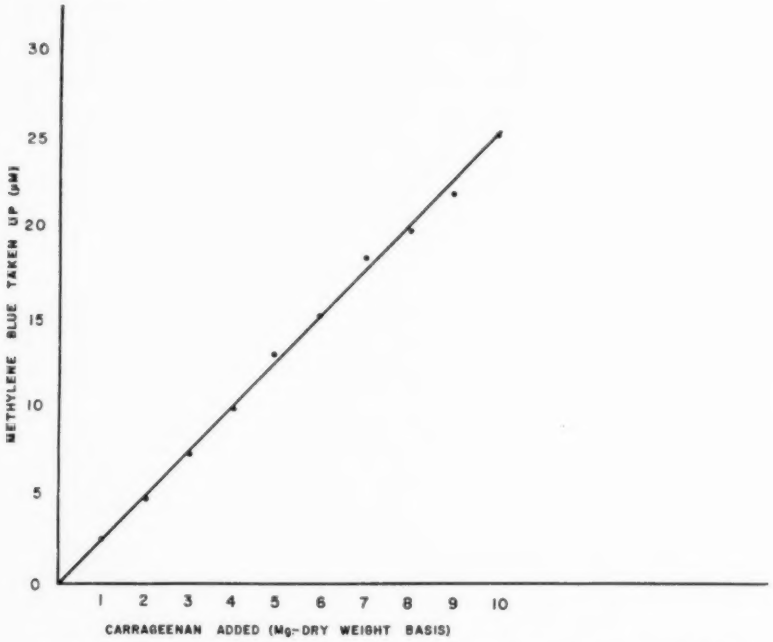

Figure 3. Uptake of methylene blue by carrageenan—Seakem type 3—undialyzed.

RECOVERY OF ADDED METHYLENE BLUE

Controlled hydrolysis of the precipitate formed through the interaction of carrageenan and methylene blue should result in release of the dye and hydrolytic products of the gum. Provided these hydrolytic products do not interfere, it should be possible to recover the methylene blue added to such systems. Therefore, experiments were designed to test this hypothesis.

To ascertain if methylene blue could be released from the interaction product, 5.6 μM of methylene blue were mixed with 4 mg of carrageenan type 3 to form a precipitate. Ten ml of 85% ortho-phosphoric acid were added and the mixture allowed to stand overnight in a water bath at 30° C. As controls, the same amount of methylene blue was added to distilled water

TABLE 2

Uptake of methylene blue by carrageenan samples

Carrageenan added	Methylene blue taken up various samples μM							
mg (dry wt basis)	Type 3		Type 402		Type 5		Type 7	
	Not Dialyzed	Dialyzed	Not Dialyzed	Dialyzed	Not Dialyzed	Dialyzed	Not Dialyzed	Dialyzed
1.0	2.50	2.53	2.72	2.66	2.99	2.72	2.99	2.77
2.0	4.89	5.06	5.03	4.89	6.12	6.08	6.03	6.16
3.0	7.47	7.61	7.88	8.00	8.16	8.16	9.00	8.92
4.0	9.51	9.79	9.79	9.52	10.88	10.60	11.83	12.03
5.0	12.45	12.51	12.64	12.50	12.91	12.91	13.00	12.92
6.0	14.95	14.95	15.07	15.00	15.63	15.60	15.72	16.86
7.0	17.67	17.70	18.13	18.21	18.35	18.76	18.67	18.14
8.0	19.30	19.52	20.66	20.39	20.39	20.39	21.07	20.40
9.0	21.75	21.74	22.57	22.43	23.11	23.52	22.72	22.43
10.0	24.46	24.20	24.20	24.74	25.83	25.83	25.99	25.83

and to 10 ml of 85% phosphoric acid and the mixtures incubated in the same way. Each mixture was then made up to 100 ml with distilled water and the methylene blue concentrations determined as outlined under the general procedure. Table 3 shows that the hydrolytic products of carrageenan did not interfere in the determination of methylene blue by the prescribed method. The per cent transmission at 650 mμ indicates that the dye was, within experimental error, completely released from the reaction product and that decomposition, if any, was minimal.

RECOVERY PROCEDURE

In the recovery procedure, 2.8 μM, 5.6 μM and 11.2 μM, respectively, of methylene blue were mixed with 4 mg of carrageenan type 3 in 15 ml centrifuge tubes. Five ml of 0.1 M $(NH_4)_2HPO_4 - NH_4OH$, pH 9.0 buffer were added and the mixtures incubated at 30° C for 12 hr. The tubes were then centrifuged at 2500 rpm, the supernatants carefully poured off, recentrifuged to remove any traces of precipitate, and the precipitates recombined. The supernatants were placed in 100 ml volumetric flasks and made up to volume with distilled water. Ten ml of 85% H_3PO_4 were added to the precipitate in each tube and a small amount of distilled water and a glass rod used to wash down any precipitate on the sides of the tubes. After 6 hr at room temperature (28 ± 1° C), the contents of each tube were transferred to a 100 ml volumetric flask and made up to volume with distilled water. After appropriate dilution, the quantity of methylene blue in the supernatant and that associated with the precipitate were determined by the usual procedure. Controls of methylene blue plus acid and methylene blue in distilled water were included. The results summarized in Table 4 show that high recovery of the added methylene blue was achieved at all added levels.

DISCUSSION AND SUMMARY

The data presented indicate that, at pH 9.0 in 0.05 M $(NH_4)_2HPO_4 - NH_4OH$ buffer, the several commercial types of carageenan take up methyl-

TABLE 3

Influence of hydrolysis product(s) of carrageenan on the absorption spectrum of methylene blue

Wave length	M.B. + Gum + H_3PO_4	M.B. + H_3PO_4	M.B. + H_2O
	Per cent transmission		
mμ			
500	93.8	94.0	94.0
520	93.0	93.2	93.0
540	89.0	89.2	88.5
560	81.0	80.8	80.0
580	70.5	70.6	70.5
600	56.5	56.0	55.0
620	46.5	46.6	45.8
640	36.5	36.2	35.8
650	34.0	34.2	33.4
655	37.0	36.5	36.0
660	39.0	39.0	39.0
680	56.5	55.5	55.0
700	80.0	80.2	79.0

ene blue in a quantitative manner. Inasmuch as dialysis had no detectable effect on the uptake of the dye, it can be concluded that the polysaccharide itself was involved in an ionic interaction with the positively charged dye. This was further substantiated by the studies on pH, where maximum uptake was realized in the alkaline media. Under the experimental conditions employed, the difference in methylene blue uptake offered no reflections on any particular traits (if any) of the commercial samples. The consistent linearity obtained for both dialysed and undialysed samples by plotting the amount of dye taken up as a function of added carrageenan, suggests strongly that with adequate control the method can be used for the quantitative estimation of carrageenan. Such usage would provide a one-step process for both identification and estimation since the reaction constitutes the official confirmatory test *(7)*. Such an application should be preceded by through dialysis of the gum if previous separation from highly heterogenous or formulated melieu is involved. More important, however, is the possibility of its application in fundamental studies on the interaction of carrageenan with chemically defined entities.

TABLE 4

Recovery of methylene blue from experimental systems

System	Methylene blue		% Recovery
	Added	Recovered	
	μM	μM	
Methylene Blue + Carrageenin	2.8	2.60	92.9
	5.6	5.45	97.1
	11.2	11.00	98.2
Methylene Blue + H_3PO_4	2.8	2.58	92.1
	5.6	5.36	95.7
	11.2	10.86	96.9
Methylene Blue in distilled water	2.8	2.65	97.0
	5.6	5.49	98.0
	11.2	10.98	98.0

Confirmatory evidence has been obtained that furcellaran, a sulfated polysaccharide closely related to carrageenan, gives the same type of fibrous precipitate with methylene blue. On a structural basis, it is expected that hypnean, a seaweed extract somewhat similar to carrageenan would also give a similar type of precipitate. Samples of this latter polysaccharide were not available for testing. For these reasons, therefore, the proposed method could, if the necessity arises, be extended to the quantitative determination of other carrageenan-like extracts. This is evident from the fact that Seakem type 402, a refined extractive from a seaweed other than Chondrus, probably an extract of Gigartina, gave quantitative relationships.

Carrageenans from different seaweeds and from different manufacturing sources may vary. Since all possibilities were not exhausted, there exists the possibility that different quantitative reactions with methylene blue may occur.

However, within a single sample or batch, reproducibility should be achievable with the recommended procedure.

The use of 70% acidified alcohol as the diluent in color intensity measurements is of special advantage. Since the gum is insoluble in alcohol, this permits a ready check on the completeness of the centrifugation step. For alternates, it was observed that acetate, citrate or phthalate buffers could also serve as diluent, provided the wave length of maximum absorption of methylene blue in such solutions was previously established.

CONCLUSIONS

In 0.05 M $(NH_4)_2HPO_4$ – NH_4OH buffer of pH 9.0, methylene blue is quantitatively taken up by carrageenan. This reaction can be used for the quantitative assay of carrageenan, provided interfering substances are previously removed. Inorganic ions and other small molecules can be removed by dialysis. Maximum uptake of methylene was shown to occur after 2 hours at 30° C and remained essentially the same after as long as 12 hours. This, along with further studies with related dyes and other gums, may help in elucidating the mechanism of action of the unique formation of the methylene blue-carrageenan precipitate and, as suggested by Stoloff *(8)*, perhaps throw some light on any yet unrecognized uniqueness in the configuration of carrageenan.

Acknowledgment

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EXAMINATION OF LEMON OIL BY GAS-LIQUID CHROMATOGRAPHY. III.[a] THE OXYGENATED FRACTION[b]

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Examination by means of gas-liquid chromatography (GLC) of the intact essential oil from the lemon reveals the necessity for deterpenation *(4, 9)* or temperature programming *(4, 5)* in order to improve resolution of the components comprising the oil. This has been discussed previously by Clark and Bernhard *(4)* in the second paper in this series. Bernhard *(3)* has shown that some 96% of the constituents present in cold-pressed California lemon oil are terpene hydrocarbons. Loading a gas chromatographic column with a whole lemon oil sample will produce a number of large peaks on a chromatogram that are due to these hydrocarbon compounds. The remaining 4% of the oil, the oxygenated compounds consisting of esters, aldehydes, ketones, etc., will produce only small peaks (in most instances due to their low relative concentration, they may not be detected at all by conventional katharometer-type detectors). Heavier loading of the column, in order to raise the level of concentration of the oxygenated compounds, severely taxes the column capacity and results in a great loss in column efficiency and distortion of detector response *(8)*. Removal of the terpene hydrocarbons from the whole oil by means of a modified Kirchner-Miller chromatographic deterpenation procedure *(9)* results in the concentration of the oxygenated compounds fraction (oxy fraction). When this oxy fraction is subjected to gas-liquid chromatographic examination, a number of peaks appear on the chromatogram which were not evident on examination of the whole oil. Thus, the minor fraction has been freed from the masking effects of the terpene hydrocarbon fraction. It may be noted that this type of separation and enrichment procedure produces an even more dramatic effect in chromatograms of orange and grapefruit oils.

In addition to the three ester-type stationary liquid phases, LAC-1-R296 (the adipate polyester of diethylene glycol); LAC-2-R446 (the adipate polyester of diethylene glycol partially cross-linked with pentaerythritol) *(10)* and methyl abietate used in the previous study *(4)*, two other ester-type liquid phases, LAC-4-R777 (the succinate polyester of diethylene glycol) and DEGS (diethylene glycol succinate), were employed in this study *(11)*. Use of these latter two liquid phases shows some improvement in the separation of the

[a] For the first and second papers in this series see: Bernhard, R. A., Examination of lemon oil by gas partition chromatography. *Food Research*, **23**, 213–216 (1958) and Clark, J. R. and Bernhard, R. A., Examination of lemon oil by gas-liquid partition chromatography. II. The hydrocarbon fraction. *Food Research*, **25**, pages 389-394 (1960).

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components of the oxy fraction over that shown by use of LAC-1-R296 and methyl abietate. Therefore these two stationary liquid phases were not employed in this study.

EXPERIMENTAL

A description of the apparatus employed to separate the constituents of the oxy fraction of lemon oil and the procedure for the modified Kirchner-Miller deterpenation have been given in detail previously *(4)*.

Separations were carried out with the aid of 3 stationary liquid phases: LAC-2-R446, LAC-4-R777, and DEGS. The materials were applied to the solid support (Sil-O-Cel C-22; 30-60 mesh) in the amount of 20% w/w. The parameters of operation for both types of LAC columns were as follows: column temperature 150°; helium flow rate 90 ml/min; sample volume 5 to 20 mμ; recorder chart speed 30 inches/hour; columns were constructed of stainless steel tubing 10 feet in length and ¼ in OD When the DEGS column was used, it was noted that the compounds eluted from the column much faster than they did when the LACs were employed as the stationary liquid phases; consequently, the operating temperature was reduced to 112° and the helium flow rate increased to 140 ml/min, and in this instance, the column length was reduced to 6 feet. The recorder chart speed was left unchanged. The column outlet was in all cases maintained at atmospheric pressure.

The lemon oil samples examined were cold-pressed California oils obtained from the Sunkist Growers, Inc., of Los Angeles, California. They were from last year's crop of fruit (April-May 1958) and were so-called raw oil samples (all waxes not removed). No antioxidants were added to the oil samples. A typical oil had the following physical properties: Specific Gravity 25°/25° = 0.852; citral content = 3.40% (actually measured as total aldehyde content); $A_D25° + 55.33°$; $n_D20°$ 1.4749.

RESULTS AND DISCUSSION

Employing LAC-2-R446 columns, gas-liquid chromatographic analysis of the oxy fractions from numerous cold-pressed California lemon oils revealed that there are 37 distinct major peaks evident on the chromatograms (Figure 1); 30 of these peaks have been numbered for purposes of identification. It may be seen that there are 7 small peaks near the origin of the chromatogram which are not numbered. The first of these peaks is due to the solvent, ethanol, that was used to elute the oxy fraction from the silicic acid. The remainder of these small peaks differ in both position—that is, retention distance—and amount from sample to sample, and have not as yet presented any consistent pattern. The peaks numbered from 1 through 30 differ generally only in amount from sample to sample. Thus the principal investigations and identifications described here were concerned with these peaks or components (work is currently in progress on the identification of the first seven or so peaks in various oil samples). Determination of the corrected retention volumes (V_R^o) *(1)* of known compounds and comparison of these with those of the unknown peaks permitted a tentative identification of a large number of the components present in this fraction. Confirmation of these results was obtained by an enrichment procedure in which known compounds were added one at a time to fresh portions of the oxy fractions and re-examined by GLC. Data are presented in the form of relative retention volumes (V_R^o/V_R^o) *(1)* (Table 1). It should be noted that it is experimentally impossible to distinguish between two compounds whose relative retention volumes differ by 7% or less since they will appear on the chromatogram as a single, united peak; differences of 10 to 15% in relative retention volumes will show peaks that are partially united, *e.g.*, shoulders, or doublets; and differences of 20% or more are necessary for complete separation of zones or peaks *(2, 7)*.

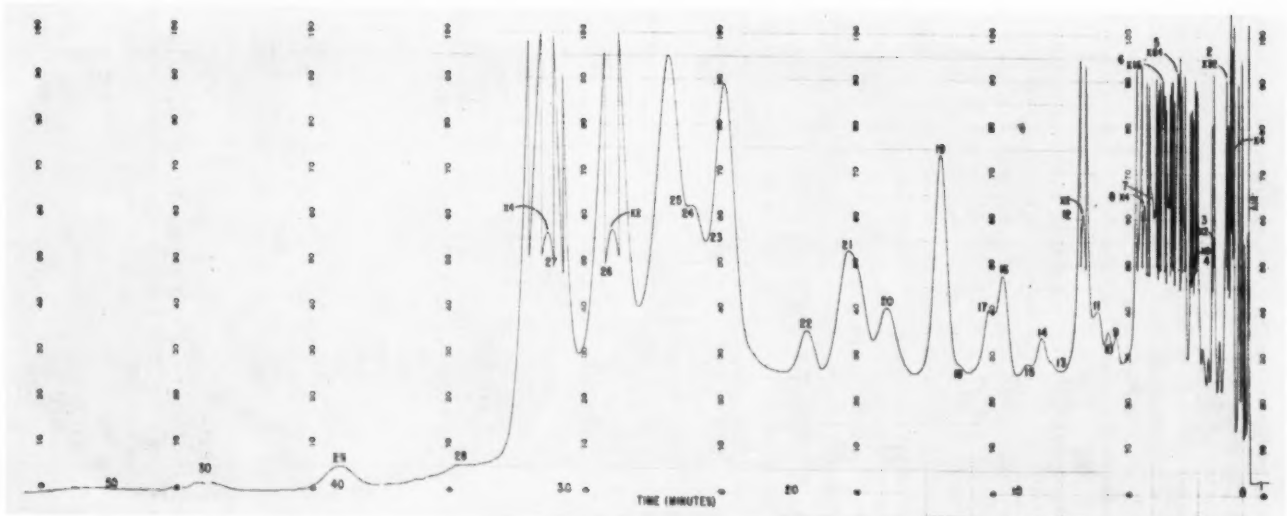

Figure 1. Gas-liquid chromatogram of the components of the oxy fraction of a cold-pressed California lemon oil. Sample size: 10 μl; temperature: 150°; helium flow rate: 90 ml/min; stationary phase: LAC-2-R446 on a support of Sil-O-Cel C-22 (30-60 mesh), 20% w/w; stainless steel column 10 ft by ¼ inch od; 1 mv recording potentiometer; chart speed: 30 inches/hour. Peak identities are presented in Table 1.

In order to be well within the bounds of experimental error, an arbitrary limit of agreement not to exceed 2% was established. Compounds with values for corrected relative retention volumes not agreeing to within 2% of each other are enclosed in parentheses. In Table 1, those peaks that differ by more than the 2% limit of agreement are: peak (5) limonene (3.0% difference); peak (20) citronellal (2.8% difference); and peak (22) decyl acetate (2.2% difference). These differences are well within the 7% limit found by James (7) and Bernhard (2).

Relative retention volumes were evaluated on a second stationary liquid phase, LAC-4-R777 (Table 2). Employing a liquid phase of LAC-4-R777, the oxy fraction showed 35 peaks. As was the case with LAC-2-R446, there were 6 small peaks near the origin that varied from sample to sample. Consequently, 29 major peaks were numbered for identification purposes. Peak identities were assigned on the basis of agreement of corrected relative retention volumes for the unknown peaks with those values for known compounds.

TABLE 1

Relative corrected retention volumes of the components in the oxy fraction of lemon oil. Stationary phase: LAC-2-R446 [1]
(*n*-decanal = 1.00)

	$V_R°/V_R°$		
Peak	Unknown	Known	Compound
1	0.065		
2	0.090		
3	0.148	0.148	α-pinene
4	0.224	0.227	β-pinene
5	0.289	0.298	limonene
6	0.354	0.351	cineole
		0.358	γ-terpinene
7	0.397		
8	0.433	0.435	p-cymene
9	0.545	0.537	3-hexen-1-ol
10	0.574	0.571	methyl heptenone
11	0.617	0.621	linalyl acetate
12	0.675	0.676	*n*-nonanal
13	0.809		
14	0.845	0.840	octyl acetate
15	0.910		
16	1.00	1.00	*n*-decanal
17	1.06	1.06	linalool
18	1.18	1.18	citronellyl acetate
19	1.25		
20	1.47	1.47	*n*-undecanal
21	1.62	1.61	geranyl formate
22	1.79	(1.83)	(decyl acetate)
23	2.13	2.17	*n*-dodecanal
24	2.25	2.23	*n*-decanol
		2.28	α-terpineol
25	2.35	2.38	citronellol; neral
26	2.58	2.61	geranyl acetate
27	2.85	2.89	citral
28	3.15	3.10	D-carvone
29	3.68	3.63	*trans*-carveol
30	4.22	4.24	geranyl butyrate

[1] Temperature 150°; helium flow rate 90 ml/min.

TABLE 2

Relative corrected retention volumes of the components in the oxy fraction of lemon oil, Stationary phase: LAC-4-R777 [1]
(n-decanal = 1.00)

	$V_R°/V_R°$		
Peak	Unknown	Known	Compound
1	0.091	.....	.....
2	0.116	.....	.....
3	0.136	0.136	α-pinene
4	0.172	.....	.....
5	0.187	.....	.....
6	0.220	0.217	β-pinene
7	0.298	0.294	limonene
8	0.354	(0.343)	(γ-terpinene)
		(0.363)	(cineole)
9	0.417	.....	.....
10	0.444	0.444	p-cymene
11	0.495	0.495	n-octanal
		0.500	n-hexanol
12	0.571	0.575	3-hexen-1-ol
13	0.697	0.701	n-nonanal
		(0.715)	(methyl-heptenone)
14	0.864	0.849	octyl acetate
15	0.899	.....	.....
16	1.00	1.00	n-decanal
17	1.08	1.07	citronellal
18	1.16	1.15	linalool
		1.18	linalyl acetate
19	1.26	.....	.....
20	1.40	1.41	linalyl propionate; n-undecanal
21	1.77	(1.71)	(decyl acetate)
		1.80	citronellyl acetate
22	1.86	.....	.....
23	2.16	(2.10)	(n-decanol)
24	2.52	(2.46)	(citronellol)
25	2.77	2.74	geranyl acetate
26	3.25	3.22	citral
27	3.68	3.68	D-carvone
28	4.27	.....	.....
29	4.60	.....	.....

[1] Temperature 150°; helium flow rate 90 ml/min.

Those peaks that differ by more than the 2% limit of agreement are: peak (8) cineole (2.5% agreement); γ-terpinene (3.2% agreement); peak (13) methyl heptenone (2.5% agreement); peak (21) decyl acetate (3.5% agreement; peak (23) n-decanol (2.9% agreement); and peak (24) citronellol (2.4% agreement); geraniol and α-terpineol (4.1% agreement). Once again these differences are well within the 7% limit (2, 7).

As an additional check on identity, relative retention volumes were evaluated on a third stationary liquid phase, DEGS (Table 3). These data also tend to reinforce the assignment of peak identities.

Employing DEGS as a stationary liquid phase, 29 major and 6 minor peaks were observed. For purposes of identification the 29 major peaks were numbered (Table 3). Those peaks that differ by more than the 2% limit of agreement are: peak (3) α-pinene (3.9% agreement); peak (8) γ-terpinene

(2.8% agreement); and peak (11) 3-hexen-1-ol (2.9% agreement). Again these differences are well within the 7% limit found by Bernhard *(2)* and James *(7)*.

It may be noted that some of the terpene hydrocarbons are still present in the oxy fraction. However, it does not appear essential that they be completely removed in order to raise the concentration level of the oxy compounds and achieve detection.

The use of 3 different stationary liquid phases lends credence to the assignment of peak identities by the methods described above. Good correlation was achieved between all 3 sets of corrected relative retention volume data obtained from the use of 3 stationary liquid phases for the following compounds: α-pinene, β-pinene, limonene, γ-terpinene, p-cymene, 3-hexen-1-ol, *n*-nonanal, methyl heptenone, octyl acetate, *n*-decanal, linalool, *n*-undecanal, citronellyl acetate, geranyl acetate, and citral. Those compounds that correlate well with two sets of corrected relative retention volume data were: *n*-octanal, cineole, linalyl acetate, linalyl propionate, decyl acetate, *n*-decanol, α-terpineol, citronellol, and D-carvone.

TABLE 3

Relative corrected retention volumes of the components in the oxy fraction of lemon oil. Stationary phase: DEGS[1]
(*n*-decanal = 1.00)

	$V_R°/V_R°$		
Peak	Unknown	Known	Compound
1	0.0510		
2	0.0669		
3	0.0860	0.0895	α-pinene
4	0.120		
5	0.152	0.154	β-pinene
6	0.169	0.171	*n*-hexanal
7	0.223	0.224	limonene
8	0.289	(0.281)	(γ-terpinene)
9	0.357	0.356	p-cymene
10	0.414	0.413	*n*-octanal
11	0.505	(0.520)	(3-hexen-1-ol)
12	0.537		
13	0.612	0.603	methyl heptenone
14	0.647	0.646	*n*-nonanal
15	0.722		
16	0.842		
17	0.885	0.882	octyl acetate
18	1.00	1.00	*n*-decanal
19	1.16		
20	1.28	1.28	linalool
21	1.40		
22	1.58	1.55	*n*-undecanal
23	1.74	1.71	linalyl-propionate
24	1.86		
25	2.26	2.23	citronellyl acetate
26	2.82	2.77	α-terpineol
27	3.27	3.25	terpinyl propionate
28	3.69	3.70	geranyl acetate
29	3.99	3.94	citral

[1] Temperature 112°; helium flow rate 140 ml/min.

A rather detailed and extensive investigation of the chemistry of cold-pressed California lemon oil conducted in the early 1930's by Poore *(12)* revealed the presence of the following compounds: α-pinene, β-pinene, D-limonene, γ-terpinene, citral, *n*-octanal, *n*-nonanal, geraniol, linalool, a tertiary alcohol (?), a crystalline aldehyde, acetic, capyrlic, and caproic acids, bisabolene, and cadinene. He was unable to detect any methyl heptenone in California oils, although this compound was reported to occur in Italian lemon oils by workers at Schimmel & Co. *(6)*.

More recently there have been extensive investigations carried out on Italian lemon oils which show the presence of two additional aliphatic aldehydes, *n*-decanal and *n*-dodecanal *(6)*. In addition Doebner *(6)* and later von Soden and Rojahn *(6)* found the terpene aldehyde citronellal. Umney and Swinton *(6)* have reported the occurrence of two esters, linalyl acetate and geranyl acetate; and workers at Schimmel & Co. have reported the presence of the terpene alcohol α-terpineol. Bohnsack *(6)* has characterized citronellol and nerol (probably present as the acetates) in Messina lemon oil.

The results of the investigations described herein agree well with the above findings. In addition to confirming those compounds reported by Poore *(12)* and Umney and Swinton *(6)*, a number of other esters were detected. The presence of octyl acetate, linalyl propionate, decyl acetate, and citronellyl acetate appear quite probable. It may well be that Poore's methods of isolation, *i.e.*, steam distillation and hydrolysis, etc., did not permit recovery of any appreciable amounts of intact ester components present in the oils.

Additionally, the results of our investigations indicate the presence of the ketone methyl heptenone, the aliphatic aldehyde, *n*-undecanal, and the aromatic hydrocarbon p-cymene (Tables 1–3). This latter compound may well not be present in true lemon oil, but might arise as an artifact after contact of the oil with silicic acid during the deterpenation procedure.

SUMMARY

The oxygenated fraction of cold-pressed California lemon oil was examined by means of gas-liquid chromatography. A number of the components present were tentatively identified by means of correlation with the corrected relative retention volumes of known compounds with those of the unknown compounds. Three different stationary liquid phases were employed in order to lend credence to the assignment of peak identities. Good correlation was achieved between all three sets of data obtained from the three stationary liquid phases for the following compounds: α-pinene, β-pinene, limonene, γ-terpinene, p-cymene; 3-hexen-1-ol, *n*-nonanal, methyl heptenone, octyl acetate, *n*-decanal, linalool, *n*-undecanal, citronellyl acetate, geranyl acetate, and citral. Those compounds that correlate well with two sets of data were: *n*-octanal, cineole, linalyl acetate, linalyl propionate, decyl acetate, *n*-decanol, α-terpineol, citronellol, and D-carvone.

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COLOR CHANGES IN PIGMENT-FREE RESIDUES FROM GAMMA-IRRADIATED GREEN BEANS, BROCCOLI, SWEET POTATOES AND CARROTS[a]

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Color changes in the pigment-free residues from several heat processed fruits and vegetables have been investigated in some detail *(2, 3, 4, 5)*. The major change is a darkening produced by interactions between the food components during and after heat treatment. It would seem that similar products treated with gamma rays might undergo reactions in common with those affecting heat processed foods, and the tissues of the "cold sterilized" products could also be expected to darken.

The present study was undertaken to investigate the effects of a number of packing and storage conditions on the color of the pigment-free residues from green beans, broccoli, sweet potatoes and carrots. The data for pigment content and color of these vegetables have been published in previous papers *(1, 6)*.

MATERIALS AND METHODS

Preparation of samples. Samples of green beans and broccoli prepared by Wishnetsky *(6)* and sweet potatoes and carrots prepared by Francheschini *(1)* were used in this study. Packing conditions and treatments are listed in Table 1 and have been described in the previous publications *(1, 6)*. All vegetables were irradiated with 1.86 megarads at the Argonne National Laboratories, Lemont, Ill.

The residues remaining after the pigments were extracted from green beans and broccoli with acetone, and from sweet potatoes and carrots with ethanol, were air-dried overnight and placed in stoppered glass vials. The vials were stored at 36–40° F until the color could be measured.

Measurement of color. The pigment-free residues were ground in a Wiley mill with a 40-mesh screen, and placed in a 2¼ inch diameter glass cell with an optical glass bottom for measurement with a Gardner Color-Difference Meter.[e] The residues were moistened with acetone *(2)* before the color readings were taken. Small area illumination with an ebonite plate having a circular hole ½ inch in diameter was employed. The instrument was standardized against a tile with color attributes Rd = 31.1, a = +0.4, b = −3.4.

The small sample size (10 g) used for pigment extraction necessarily resulted in a small amount of residue. This small amount of residue did not introduce undue difficulties in color measurement as it was possible to pack the residue in the center of the cell. The possibility that a larger sample size would change the color readings was tested by measuring 36 sets of duplicate residue samples from green beans and broccoli, ranging from 30 to 160 cu mm, individually and then after combining the samples.

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TABLE 1

Experimental treatments for green beans, broccoli, sweet potatoes and carrots

Treatment no.	Irrad temp	pH	Can lining	Packing medium	Headspace gas	Storage temp
1	Room temp	Normal	Plain tin	Brine	Vacuum	Room temp
2[1]	Room temp	High	Plain tin	Brine	Vacuum	Room temp
3	Room temp	Normal	Enameled	Brine	Vacuum	Room temp
4[1]	Room temp	High	Enameled	Brine	Vacuum	Room temp
5	Room temp	Normal	Enameled	Brine	Nitrogen	Room temp
6	Room temp	Normal	Enameled	Water	Vacuum	Room temp
7	Room temp	Normal	Enameled	Dry	Vacuum	Room temp
8	Room temp	Normal	Enameled	Dry	Nitrogen	Room temp
9	Room temp	Normal	Enameled	Dry	Air	Room temp
10	Room temp	Normal	Enameled	Brine	Vacuum	100° F
11	Frozen	Normal	Plain tin	Brine	Vacuum	Room temp
12[1]	Frozen	High	Plain tin	Brine	Vacuum	Room temp
13	Frozen	Normal	Enameled	Brine	Vacuum	Room temp
14[1]	Frozen	High	Enameled	Brine	Vacuum	Room temp
15	Frozen	Normal	Enameled	Brine	Nitrogen	Room temp
16	Frozen	Normal	Enameled	Water	Vacuum	Room temp
17	Frozen	Normal	Enameled	Dry	Vacuum	Room temp
18	Frozen	Normal	Enameled	Dry	Nitrogen	Room temp
19	Frozen	Normal	Enameled	Dry	Air	Room temp
20	Frozen	Normal	Enameled	Brine	Vacuum	100° F
21	Frozen	Normal	Enameled	Brine	Vacuum	0° F
22	Non-irrad Control	Normal	Enameled	Brine	Vacuum	0° F

[1] The high pH samples refer only to green beans and broccoli.

A comparison of the readings indicated no change in Rd, a, or b as a result of the increased sample size. The sweet potato and carrot samples presented no difficulty because the residues were present in larger quantities.

Another representative set of 42 samples from green beans and broccoli was extracted with acetone and ethanol in order to determine whether the extractant medium influenced the color of the residues. The ethanol extracted samples had slightly higher Rd values than the acetone extracted samples but the "a" and "b" values were identical.

The color readings were interpreted by using Rd as an index of darkening, $\tan^{-1}a/b$ for hue and $(a^2+b^2)^{1/2}$ for chroma. The conclusions were based on analyses of variance for seven groupings at the one per cent level of significance.[f]

RESULTS AND DISCUSSION

Over-all effects of irradiation and storage

Green Beans. The reflectance values for the non-irradiated frozen controls (Rd = 25.6) were appreciably higher after 3 weeks of storage than those of any of the irradiated samples. This suggests that the reactions contributing to the discoloration were active either during or soon after the irradiation period. The darkening of the residues (Table 2) continued during the first 15 weeks of storage and then levelled off. The hue of the frozen controls ($\tan^{-1}a/b = 5.0$) was similar to the irradiated samples and showed a gradual decrease up to 25 weeks of storage. This color change may best be described as a small change from brown toward yellow. The chroma of the frozen controls [$(a^2+b^2)^{1/2} = 12.6$] was similar to the irradiated samples and showed little change on storage.

[f] In the interest of brevity, tables of statistical data were not reproduced here. Tables of analyses of variance will be supplied by the authors upon request.

Broccoli. Reflectance values for non-irradiated controls (Rd = 14.1) were higher at the two-week storage period than the irradiated samples, but the effect was less marked than with green beans. The tissues darkened slowly over the whole storage period (Table 2). Hue values for controls were much lower (1.8) than for irradiated samples, and increased markedly with storage up to 25 weeks and then decreased. This change was the reverse of that occurring in green beans. Chroma values for controls (16.8) were lower than the initial values for irradiated samples but the latter levelled off at approximately 16 after 5 weeks of storage.

Carrots. Reflectance values for controls (35.1) were much higher than the initial values for irradiated samples, and the latter decreased considerably on storage (Table 2). The hue of the controls (2.6) was much lower than the irradiated samples after the three week storage period, and the change continued until the 8th week, and then leveled off. At this point, the color of the residues might be described as having shifted from yellow toward brown. The chroma of the controls (10.5) was lower than that of irradiated samples and the latter continued to rise for 13 weeks of storage, and then decreased.

Sweet potatoes. Reflectance values for controls (30.6) were similar to the initial values for the irradiated samples but the latter decreased considerably

TABLE 2

Color of pigment-free residues from four vegetables irradiated at 1.86 megarads and stored under the conditions described in treatments 1 to 20 of Table 1

Storage time, weeks	Color					
	Reflectance Rd		Hue $\tan^{-1} b/b$		Chroma $(a^2 + b^2)^{1/2}$	
	RT[1]	F[2]	RT[1]	F[2]	RT[1]	F[2]
Green beans [3]						
3	13.8	13.5	7.0	5.4	15.2	14.2
7	8.3	9.4	6.3	5.0	13.6	13.8
15	6.5	7.6	6.8	3.8	13.0	13.5
25	6.4	7.6	3.7	2.2	11.8	13.0
48	5.0	7.1	6.4	4.3	11.6	13.2
Broccoli [3]						
2	12.8	13.2	6.1	4.8	12.3	11.6
6	9.0	11.0	9.2	7.7	15.9	16.2
16	7.8	11.7	12.0	10.5	15.8	17.1
25	7.2	9.4	13.5	11.6	15.5	16.4
46	5.6	7.2	12.4	9.4	14.5	16.0
Sweet potatoes [4]						
2	30.0	29.6	9.6	9.0	15.4	14.6
6	30.1	28.3	12.1	12.5	16.2	15.6
16	28.0	27.2	17.6	15.5	18.2	16.3
24	21.5	19.5	17.3	15.2	18.0	15.2
43	25.0	25.4	15.8	13.2	15.6	14.4
Carrots [4]						
3	23.3	25.0	10.1	7.3	17.2	15.0
6	17.0	21.9	19.6	15.5	19.3	18.2
13	12.7	16.3	20.1	14.0	19.3	19.0
25	9.0	15.0	23.0	14.3	16.9	18.7
43	12.2	17.1	19.7	17.0	16.1	16.6

[1] Irradiated at room temperature.
[2] Irradiated in the frozen state.
[3] Each datum represents the average value obtained from duplicate analyses on each of 20 cans.
[4] Each datum represents the average value obtained from duplicate analyses on each of 16 cans.

upon storage (Table 2). The hue of the controls (5.5) was much lower than the irradiated samples and the latter continued to rise until the 16th week of storage, and then decreased slowly. The chroma of controls (12.6) was lower than that of irradiated samples but little change was evident upon storage.

In Table 8, the change in color on storage of the residues from the sweet potato samples listed in Table 7 is presented. Changes in hue and chroma (reflectance, hue and chroma of the initial value for the controls were 20.2, 0.0, and 11.7, respectively) were similar but the reflectance values increased with storage. However, values for the samples shown in Table 2 were higher initially and the two were similar after 25 weeks of storage. This effect may be a result of differences in the maturity of the potatoes, as samples considered in Table 2 were packed in October and samples considered in Table 8, in March.

Effects of temperature of product during irradiation

The irradiation temperature influenced the color of the pigment-free residues from green beans as the samples irradiated frozen showed less darkening and less hue change (Table 2). Differences in chroma were not significant at the 1% level. The same conclusions are evident also for broccoli (Table 2). With carrots, the samples irradiated frozen were lighter in reflectance and showed less hue change. There was a significant interaction between storage time and chroma as the graphs cross in Table 2. Reflectance values for sweet potatoes were lower for samples irradiated frozen in contrast with those for the other 3 vegetables. There was less hue and chroma change for the samples irradiated frozen.

Effects of headspace gas

Residues from vegetables packed dry in air, vacuum, or nitrogen differed in reflectance, hue, and chroma but the changes were relatively small. Residues from green beans and broccoli packed in air showed considerable darkening (Table 3) when irradiated at room temperature but not when irradiated frozen. Samples of sweet potatoes in air had the greatest decrease in brightness, the smallest change in hue. Samples of carrot packed dry under vacuum and irradiated frozen had the darkest residues.

When the vegetables were packed in brine, syrup, or water, the headspace gas also influenced the color of the residues (Tables 4 and 5) but the changes were relatively small.

Effects of packing media

The packing medium (water, brine, or syrup) had a small but significant effect on the color of residues. With green beans (Table 4), hue and chroma were lower for the water samples irradiated at room temperature and higher for water samples irradiated frozen. The Rd, hue and chroma of the broccoli residue samples irradiated at room temperature in water were higher than the brine samples but the order was reversed when irradiated frozen. The hue and chroma of carrot tissue samples were lower in water than in brine. With sweet potato residues, all 3 color attributes were lower in water than in syrup.

TABLE 3

Effect of type of atmosphere on color of pigment-free residues in four vegetables [1]

	Irradiated at room temp			Irradiated frozen		
	Vacuum	Nitrogen	Air	Vacuum	Nitrogen	Air
Green Beans						
Reflectance [2]	8.9	8.9	6.9	8.6	8.9	9.1
Hue [2]	6.5	7.5	9.1	4.2	5.4	9.0
Chroma [2]	13.4	13.1	13.3	13.3	12.6	14.2
Broccoli						
Reflectance	9.0	7.8	7.2	10.2	8.2	9.0
Hue	9.8	12.5	11.0	8.6	10.4	9.2
Chroma	14.0	14.5	13.7	14.8	15.9	14.7
Sweet Potatoes						
Reflectance	26.1	27.9	25.2	26.3	23.9	23.0
Hue	12.3	12.6	12.1	12.6	12.6	11.9
Chroma	15.8	16.2	15.9	14.7	14.9	14.4
Carrots						
Reflectance	18.2	18.9	18.2	17.5	19.5	20.4
Hue	17.9	17.9	18.3	16.0	14.4	14.9
Chroma	17.9	17.9	18.0	17.0	17.7	18.5

[1] The vegetables were packed dry in enameled cans and stored at room temperature. Each datum was obtained from an average of 10 cans, i.e., 2 at each of the 5 storage periods listed in Table 2.
[2] The reflectance, hue and chroma were represented by Rd, $\tan^{-1}a/b$, and $(a^2+b^2)^{1/2}$ respectively.

Effects of can lining

The can lining [4] had a marked effect on the color of the residues (Tables 4, 5). Green beans packed in brine under vacuum had much higher tissue

[4] Cans obtained through the courtesy of the American Can Co., Maywood, Ill.

TABLE 4

Effect of packing in water vs brine on the color of pigment-free residues from four vegetables [1]

	Irradiated at room temperature		Irradiated frozen	
	Brine	Water	Brine	Water
Green Beans				
Reflectance [2]	8.0	7.7	9.0	10.1
Hue [2]	5.4	5.3	2.6	3.4
Chroma [2]	12.7	11.6	11.8	12.7
Broccoli				
Reflectance	8.5	8.0	10.7	11.9
Hue	10.9	10.5	9.1	9.8
Chroma	16.0	14.7	16.1	15.1
Carrots				
Reflectance	12.5	12.6	20.4	19.6
Hue	19.0	18.3	13.7	11.3
Chroma	17.9	17.7	17.2	16.8
Sweet Potatoes				
	Syrup	Water	Syrup	Water
Reflectance	28.8	27.7	28.3	26.9
Hue	14.6	14.5	14.4	10.9
Chroma	16.8	16.1	15.3	14.1

[1] The vegetables were packed under vacuum in enameled cans and stored at room temperature. Each datum was obtained from an average of 10 cans, i.e., 2 at each of the 5 storage periods listed in Table 2.
[2] The reflectance, hue and chroma were represented by Rd, $\tan^{-1}a/b$, and $(a^2+b^2)^{1/2}$ respectively.

reflectance and chroma values when packed in cans with a plain tin lining as opposed to R-enamel cans. Hue values were also different but the effect was complicated by interactions between storage time and irradiation temperature. Reflectance values for broccoli residues were higher with the plain tin cans particularly when irradiated at room temperature. Average values for hue and chroma were also higher for plain tin cans but these effects were not significant at the 0.05% level.

The can lining had a highly significant effect on the residues from carrots packed in brine under vacuum (Tables 4, 5). Reflectance and chroma values were higher for the samples in plain tin but hue changes were less. Hue changes for both carrot and sweet potato tissues had significant interactions with storage time and irradiation temperature. The can lining also influenced the chroma of the residues from sweet potatoes by interacting with storage time. Chroma values for the later storage periods were higher for the samples in plain tin.

The effect of the container is also shown in another experiment (Table 7). Samples in plain tin cans had much higher Rd and chroma values and yellower hues than the residues from samples in glass vials.

Effects of storage temperature

Storage temperature affected the reflectance and hue of green beans packed in brine under vacuum in enamelled cans (Table 6). Samples stored at 100° F were darker and browner than samples at 70° F. Residues from broccoli stored at 100° F were darker and browner than the samples at 70° F, but the former had lower chroma values. Residues from sweet potatoes stored at 100° F were darker, had greater hue changes (i.e. were browner) and had

TABLE 5

Effect of headspace gas and type of can lining on color of pigment-free residues from four vegetables [1]

	Plain tin cans Vacuum headspace		Enameled cans Nitrogen headspace	
	Irrad at R.T.	Irrad frozen	Irrad at R.T.	Irrad frozen
Green Beans				
Reflectance [2]	9.0	12.6	7.7	7.9
Hue [2]	4.8	3.2	6.6	2.9
Chroma [2]	14.7	16.1	12.2	12.1
Broccoli				
Reflectance	9.8	11.4	8.6	11.6
Hue	10.6	7.6	10.9	8.8
Chroma	15.2	15.6	16.4	16.1
Sweet Potatoes				
Reflectance	29.8	29.4	28.8	26.6
Hue	14.8	11.7	16.5	12.9
Chroma	18.0	16.5	16.9	15.7
Carrots				
Reflectance	14.7	23.4	15.5	19.3
Hue	18.0	10.7	18.8	12.6
Chroma	19.5	19.3	17.7	16.8

[1] The vegetables were packed in brine or syrup (sweet potatoes only) and stored at room temperature. Each datum was obtained from an average of 10 cans, i.e., 2 at each of the 5 storage periods listed in Table 2. Comparable data for packs in enameled cans under vacuum are presented in Table 4.

[2] The reflectance, hue and chroma were represented by Rd, $\tan^{-1} a/b$, and $(a^2 + b^2)^{1/2}$ respectively.

TABLE 6

Effect of storage temperature after irradiation on the color of pigment-free residues from four vegetables [1]

	Storage temperature				
	0° F	70° F		100° F	
	Irrad frozen	Irrad at R.T.	Irrad frozen	Irrad at R.T.	Irrad frozen
Green Beans					
Reflectance [2]	11.9	8.0	9.0	5.8	7.7
Hue [2]	3.5	5.4	2.6	7.5	5.4
Chroma [2]	13.0	12.7	11.8	11.6	12.2
Broccoli					
Reflectance	15.5	8.5	10.7	6.8	8.7
Hue	3.7	10.9	9.1	12.4	11.8
Chroma	13.9	16.0	16.1	13.5	15.4
Sweet Potatoes					
Reflectance	32.6	28.8	28.3	21.8	24.1
Hue	9.3	14.6	14.4	19.0	17.2
Chroma	14.2	16.8	15.3	17.7	16.3
Carrots					
Reflectance	25.4	12.5	20.4	9.1	14.3
Hue	6.6	19.0	13.7	21.1	15.9
Chroma	15.4	17.9	17.2	15.8	16.9

[1] The vegetables were packed in brine or syrup (sweet potatoes only) under vacuum in enameled cans. Each datum was obtained from an average of 10 cans, i.e., 2 at each of the 5 storage periods listed in Table 2.

[2] The reflectance, hue and chroma were represented by Rd, $\tan^{-1}a/b$, and $(a^2 + b^2)^{1/2}$ respectively.

higher chroma values than samples at 70° F. Residues from carrots stored at 100° F were much darker and showed greater hue changes, but smaller chroma changes than samples stored at 70° F. Samples from both carrots and sweet potatoes stored at 0° F were lighter and had smaller changes in hue and chroma.

Effects of blanching and puréeing

Effects of blanching and puréeing sweet potatoes on the color of the pigment-free residues is reported in Table 7. The effects of blanched versus

TABLE 7

Color of the pigment-free residues from gamma-irradiated sweet potatoes which had been blanched, puréed or chemically treated

Treatment	Reflectance Rd	Hue $\tan^{-1}a/b$	Chroma $(a^2 + b^2)^{1/2}$
1. Sliced, unblanched, plain tin cans	22.1 [1]	9.2	17.6
2. Sliced, blanched, plain tin cans	23.1	6.8	17.8
3. Sliced, unblanched, plain tin, KCN dip	27.3	9.7	17.8
4. Puréed, unblanched, plain tin cans	24.6	7.9	17.1
5. Puréed, blanched plain tin cans	25.8	0.1	13.4
6. Puréed, unblanched, plain tin, KCN added	27.7	9.1	17.8
7. Puréed, blanched, glass vials	19.7	5.8	14.0
8. Puréed, unblanched, glass vials	21.7	8.7	14.0
9. Puréed, unblanched, glass vials, chlorogenic acid	10.6	11.6	14.5
10. Non-irradiated, sliced, unblanched, plain tin	20.9	1.8	13.6
11. Non-irradiated, sliced, blanched, plain tin	21.6	−1.9	10.1

[1] Each datum was obtained from an average of 8 cans, i.e., 2 at each of the 4 storage periods indicated in Table 8. After processing, the sweet potatoes were irradiated in the frozen state at 1.86 megarads and stored at 70-75° F. The samples in treatments 10 and 11 were stored frozen.

unblanched, and puréed versus sliced samples were similar, in that both blanching and puréeing raised the reflectance values and lowered hue and chroma values. The puréeing process would incorporate more air into the samples whereas the blanching process would inhibit any enzyme action. The color changes may be both oxidative and enzymatic in nature.

Effects of chemical additives

Addition of potassium cyanide, which had such a marked effect on the color and pigment content of sweet potatoes *(1)*, also had a considerable effect on the color of the residues (Table 7). The Rd, hue and chroma values of the residues from cyanide-treated samples were much higher than the controls, indicating the chemical had inhibited the reactions contributing to darkening but not those that changed hue and chroma.

TABLE 8

Color of the pigment-free residues from sweet potatoes irradiated and stored under the conditions described in Table 7

Storage time, weeks	Reflectance Rd	Color[1] Hue $\tan^{-1}a/b$	Chroma $(a^2+b^2)^{1/2}$
2	19.5	1.5	16.0
6	24.2	6.5	15.5
15	23.7	11.4	16.2
27	23.0	10.6	16.0

[1] Each datum represents the average obtained from duplicate analyses on each of 18 cans from Treatments 1 to 9 of Table 7.

TABLE 9

Effects of added ascorbic acid on the color of pigment-free residues from green bean and broccoli purées

Ascorbic acid per cent	Green Beans			Broccoli		
	Reflectance Rd	Hue $\tan^{-1}a/b$	Chroma $(a^2+b^2)^{1/2}$	Reflectance Rd	Hue $\tan^{-1}a/b$	Chroma $(a^2+b^2)^{1/2}$
0.00[2]	18.0	4.8	11.9	21.8	0.0	16.3
0.00	18.0	7.2	14.3	21.0	4.0	17.3
0.01	18.4	7.2	14.3	21.2	2.3	17.6
0.05	17.5	10.0	13.8	20.4	5.1	16.9
0.10	16.9	13.2	14.0	19.5	4.9	17.7
0.50	21.2	7.9	15.4	22.8	2.6	17.5
0.50[2]	23.3	4.1	13.9	35.0	−0.8	13.6

[1] Non-irradiated control.
[2] Non-irradiated control with 0.50 per cent ascorbic acid added.

Chlorogenic acid, which contributed to considerable color change in sweet potatoes, also affected the color of residues (Table 7). Samples with added chlorogenic acid had residues that showed the greatest change in both Rd and hue in the experiment reported in Table 7.

The effect of added ascorbic acid on the color of residues from green beans and broccoli is reported in Table 9. Apparently, adding ascorbic acid decreased the Rd and increased the hue value for residues from green beans until 0.50% was reached, whereupon the trend was reversed. With broccoli, the same trend was evident for reflectance values but not for hue values.

TABLE 10

Ten treatments for irradiated vegetables with the least change in color of pigment-free residues

Reflectance	Hue	Chroma	Reflectance	Hue	Chroma
Green Beans			Broccoli		
12.6[1] (11)[2]	5.3 (2)	13.3 (9)	11.8 (16)	6.6 (14)	16.4 (5)
11.8 (12)	5.3 (6)	13.3 (17)	11.8 (14)	6.8 (12)	16.1 (13)
10.5 (2)	4.8 (1)	13.1 (8)	11.6 (15)	7.6 (11)	16.1 (15)
10.1 (16)	4.2 (17)	12.7 (3)	11.4 (11)	8.6 (17)	16.0 (3)
9.1 (19)	3.4 (14)	12.6 (18)	10.7 (13)	8.8 (15)	15.9 (18)
9.0 (1)	3.4 (16)	12.2 (20)	10.2 (12)	9.0 (2)	15.7 (2)
9.0 (13)	3.2 (11)	12.1 (15)	10.2 (17)	9.1 (13)	15.6 (11)
8.9 (18)	2.9 (15)	11.8 (13)	9.8 (1)	9.2 (19)	15.4 (12)
8.9 (7)	2.8 (4)	11.8 (4)	9.7 (2)	9.4 (4)	15.2 (1)
8.9 (8)	2.6 (13)	11.6 (6)	9.0 (19)	9.8 (16)	15.1 (16)
11.9 (21)[3]	3.5 (21)	13.0 (21)	15.5 (21)	3.7 (21)	13.9 (21)
15.8 (22)[3]	3.8 (22)	12.5 (22)	14.7 (22)	2.6 (22)	16.4 (22)
Sweet Potatoes			Carrots		
29.8 (1)	10.9 (16)	16.3 (20)	23.4 (11)	10.7 (11)	15.8 (10)
29.4 (11)	11.7 (11)	16.2 (8)	20.4 (13)	11.3 (16)	16.8 (15)
28.8 (3)	11.9 (19)	16.1 (6)	20.4 (19)	12.6 (15)	16.8 (16)
28.8 (5)	12.1 (9)	15.9 (9)	19.6 (16)	13.7 (13)	16.9 (20)
28.3 (13)	12.3 (7)	15.8 (7)	19.5 (18)	14.4 (18)	17.0 (17)
27.9 (10)	12.6 (17)	15.7 (15)	19.3 (15)	14.9 (19)	17.2 (13)
27.7 (6)	12.6 (8)	15.3 (13)	18.9 (8)	15.9 (20)	17.7 (18)
26.9 (20)	12.6 (18)	14.9 (18)	18.2 (9)	16.0 (17)	17.7 (5)
26.6 (19)	12.9 (15)	14.7 (17)	18.2 (7)	17.9 (7)	17.7 (6)
26.3 (17)	14.5 (6)	14.4 (19)	17.5 (17)	17.9 (8)	17.9 (3)
32.6 (21)	9.3 (21)	14.2 (21)	25.4 (21)	6.6 (21)	15.4 (21)
27.9 (22)	9.1 (22)	15.0 (22)	28.9 (22)	4.6 (22)	15.5 (22)

[1] Each datum represents the average obtained from 10 cans, i.e., 2 at each of 5 storage periods.
[2] The figures in brackets represent the treatment numbers as described in Table 1.
[3] Treatments 21 and 22 represent the irradiated and non-irradiated samples stored at zero ° F.

Optimum conditions for minimizing color change

The 10 treatments with the least change in color of the pigment-free residues from the 4 vegetables are listed in Table 10. In general, conditions that caused the least change were similar for green beans and broccoli and for sweet potatoes and carrots. This was particularly true for hue but not so much so for reflectance and chroma. Even for one vegetable, optimum treatments for reflectance were not necessarily optimum for hue or chroma. Thus, the choice of optimum treatment may depend on the effect, if any, of each color attribute of the pigment-free residues on the consumer acceptance of the original product.

Color of the product is related to color of the pigment-free residues as the two show a significant correlation (Table 11). Correlations for Rd were positive for all 4 vegetables because values for the controls were higher than all the irradiated samples for both product and residues except with green beans where there were Rd values for the product that were both higher and lower than the controls. Correlations for hue were negative for green beans, broccoli, and carrots because hue values of the product were higher than the controls and hue values for residues were lower than the controls. This meant

a treatment that produced a large hue change would lower the hue of the product and raise the hue of the residue. Correlations for chroma were positive for the same reasons as given for reflectance. Chroma values of the controls for the product were higher than the treatments for green beans, broccoli, and carrots and midway for sweet potatoes. Chroma values of residues from the controls were higher than the irradiated samples for broccoli and carrots, and midway for green beans and sweet potatoes. Hue values for sweet potatoes did not correlate, probably because of the very active enzyme systems that contributed to the color of the product.

TABLE 11

Correlations between color attributes of the product and those of the pigment-free residues

	Reflectance	Hue correlation[1] (r)	Chroma
Green Beans	+0.660	−0.391	+0.916
Broccoli	+0.656	−0.646	+0.679
Sweet Potatoes	+0.818	+0.122	+0.529
Carrots	+0.876	−0.803	+0.480

[1] Each correlation was based on 20 sets of values for green beans and broccoli and 16 for sweet potatoes and carrots (Table 1). The figures for the five storage periods were averaged to provide one set of values.

Correlations between the color of the product and the color of the residues may be interpreted in two ways. Either the color of the product was being influenced by the color of the residues or the reactions that influence the color of the product also influenced the color of the residues. The data here do not enable one to tell whether one or both of the above postulates are true for reflectance and chroma. However, the data for hue would favor the hypothesis that the conditions which created a large change in the product also created a large hue change in the residues, albeit in the opposite direction.

SUMMARY AND CONCLUSIONS

The color of the pigment-free residues from gamma-irradiated green beans, broccoli, sweet potatoes and carrots packed under a variety of conditions and stored for one year have been investigated. The chief findings were:

1. The color of the residues from the irradiated samples was appreciably different from those from the frozen controls. Some changes occurred during or soon after irradiation and further changes occurred during storage.

2. Green bean residues after 11 months' storage were darker and showed a small hue change from the brown to the yellow. The broccoli, sweet potato, and carrot samples were all darker and browner after 11 months' storage.

3. Residues from green beans, broccoli, and carrots irradiated in the frozen state showed less darkening and less hue change than those irradiated at room temperature. Residues from sweet potatoes showed more darkening but less hue change when irradiated frozen.

4. Residues from vegetables packed dry in air were generally darker and showed more color change than the samples packed dry in vacuum or nitrogen.

5. Residues from vegetables packed in water were generally darker and showed more color change than the samples packed in brine or syrup.

6. Residues from vegetables packed in plain tin cans were lighter and showed less hue change than in enamelled cans. The color of the residues from vegetables in plain tin cans was more attractive.

7. Residues from vegetables stored at 100° F were much darker and showed more hue change than the samples at 70° F.

8. Blanching and puréeing sweet potatoes changed the color of the residues considerably. Reflectance values were higher, and the hue and chroma values were lower.

9. Addition of potassium cyanide inhibited the reactions that contributed to the darkening in sweet potato residues but not the hue and chroma. Chlorogenic acid caused considerable change in reflectance, hue and chroma.

10. Reflectance and chroma values for the product and the residues showed positive correlations for all four vegetables. The hue values showed a negative correlation for green beans, broccoli and carrots and no correlation for sweet potatoes. This indicated, in the case of hue, at least, that conditions which created a large color change in the product also created a large color change in the residues.

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BROWNING OF SUGAR SOLUTIONS. IV. THE EFFECT OF pH ON THE VOLATILE PRODUCTS OF REDUCING SUGARS[a]

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The principal steam volatile carbonyl compounds obtained from the alkaline decomposition of reducing sugars are generally considered to be methylglyoxal and acetol. These compounds have also been suggested as browning intermediates *(22, 23)*. It is still doubtful, however, as to which of these two compounds represents the major product formed in the reaction. Fernbach and Schoen *(7)*, Fischler *(8, 9)*, Evans *(6)*, and Enders *(3, 4, 5)* have implicated methylglyoxal on the basis of the isolation of its phenylosazone. However, Sattler and Zerban *(16)* have questioned the use of this technique as positive proof of the presence of methylglyoxal since methylglyoxalosazone can also be formed from acetol. Baudische and Deuel *(1)* by means of a specific reaction of acetol with o-aminobenzaldehyde have indicated that it is acetol and not methylglyoxal which is produced in the distillation of glucose from weakly alkaline solution. Similarly, Nodzu *et al (12, 13)* based on the differential solubility of the semi-carbazone of acetol and methylglyoxal, also report the production of acetol but not methylglyoxal from several reducing sugars in mildly alkaline solutions. Prey and his co-workers *(14, 15)* using paper chromatography were able to demonstrate the formation of both acetol and methylglyoxal in alkaline solutions of glucose, dihydroxyacetone and glyceraldehyde. Similarly, Sattler and Zerban *(17)* have isolated acetol and a relatively lesser amount of methylglyoxal through solvent extraction of heated aqueous fructose solution.

During our investigation on color development in maple sirup, which is thought to occur through an alkaline degradation of the hexose constituents of the sap, several low molecular weight carbonyl compounds have been isolated as their dinitrophenyl-bis-hydrazones *(21)*. In an attempt to clarify further the nature of these compounds and their role in the mechanism of color and flavor development in the sirup, the authors have for some time been conducting experiments with model systems of dilute sugar solutions. In these preliminary studies it was observed that under varied experimental conditions, particularly of pH, the volatile constituents obtained from the sugars varied in both the nature of the compounds and in the amounts produced. This suggested that the pH of the reaction could explain why either acetol or methylglyoxal have been identified as the primary steam volatile products of the degradation. To clarify the extent to which these

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[b] From work done for a thesis submitted by H. G. Lento to the Graduate School of Georgetown University as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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compounds are produced, a study was made of the effect of pH on the formation of acetol and methylglyoxal from such reducing sugars as glucose, fructose, and dihydroxyacetone. This study required a means for distinguishing and estimating acetol and methylglyoxal when present in a mixture. Of the known methods of analysis for these compounds the procedures of Speck and co-workers *(10, 20)* were found to be the most specific and sensitive available. Methylgloxal is determined fluorometrically after its reactions with chromotropic acid and sulfuric acid. Acetol is also estimated fluorometrically after its reaction with o-aminobenzaldehyde in alkaline solution. Unfortunately, diacetyl interferes in both of these methods. Therefore, since this compound had been shown to be a volatile component in the distillates of reducing sugars *(13, 19)* it was necessary to eliminate diacetyl from solutions of acetol or methylglyoxal prior to their analysis. Recently Gabrielson and Samuelson *(11)* used an ion exchange column in the bisulfite form to remove and separate aldehydes and ketones in solutions of alcohol. Based on this, a method was developed using a bisulfite ion exchange technique for the quantitative separation of acetol, methylglyoxal and diacetyl when present in a mixture. This procedure has been applied to study the effect of pH on the production of these compounds from glucose, fructose and dihydroxyacetone.

EXPERIMENTAL

Materials. Acetol, methylglyoxal and diacetyl were obtained as "pure" compounds and further purified in the laboratory by vacuum distillation. Their concentration in the distillate was determined by periodic acid oxidation *(2)* and by precipitation with 2,4-dinitrophenylhydrazine.

Glucose, fructose and dihydroxyacetone were used without further purification as were all other chemicals and reagents used in these studies.

The phosphate buffer solutions were 0.25 molar disodium hydrogen phosphate adjusted to the desired pH with either one normal sodium hydroxide or with 85% phosphoric acid.

A Lumitron[4] photofluorometer equipped with a Corning No. 5874 red-purple primary filter and two secondary filters, Corning No. 3384 and Noviol 4308, was employed for the fluorescence measurements.

A Coleman Universal Colorimeter was used for colorimetric determination of diacetyl.

Chromatographic separation of acetol, diacetyl and methylglyoxal. Three grams of the anion exchanger IR400 (60-80 mesh) were washed with water to remove the suspended solids and packed as a wet slurry in a jacketed 15 mm ID glass column to a height of 25 cm. The column was fitted with a Teflon stopcock and a plug of glass wool to retain the resin. The temperature of the resin was kept at 4° C by circulating ice water through the jacket of the column so that the carbonyl compounds could be effectively retained as the bisulfite addition product.

The resin was placed in the hydroxyl form by treatment with 50 ml of 1.0 N NaOH. After washing the resin free of alkali with distilled water, 50 ml of a freshly prepared 0.2 N sodium bisulfite solution were added and the excess reagent removed with 100 ml of water. Twenty-five ml containing a known amount of acetol, methylglyoxal and diacetyl were added to the top of the ion exchange column and passed through it at the rate of 5 drops per minute. The complete retention of the carbonyls by the ion exchange resin was checked by testing the effluent with 2,4-dinitrophenylyhdrazine.

Five hundred ml of 0.05 N sodium chloride were used to elute the carbonyls from the resin. The eluate was collected in 10 ml fractions with an automatic fraction collector. A 1 ml aliquot of each of the 10 ml eluates was removed and tested with 2,4-

[4] Mention of company and trade names does not imply endorsement by the Department over others not named.

dinitrophenylhydrazine to locate those fractions containing the carbonyls. Five ml of each of the consecutive tubes of the 3 groups giving a positive test were combined. A 1 ml aliquot was removed and tested qualitatively and quantitatively to identify and to determine the amount of compound present. The tests for methylglyoxal, diacetyl and acetol were those of Speck *(10, 18, 20)*. However, to test for acetol the bisulfite was first removed from the solution with sulfuric acid (0.03 ml). The test solution was freed from SO_2 by bubbling N_2 gas through it for several minutes. The solution was then neutralized with sodium hydroxide and the fluorometric determination of acetol performed. Table 1 shows the separation and recovery of acetol, methylglyoxal and diacetyl from mixtures obtained by the bisulfite ion exchange procedure.

TABLE 1

Separation of mixtures of carbonyls by bisulfite chromatography

Compound	Composition of mixture	Found	Recovery
	mg	*mg*	%
Acetol	2.00	1.92	96.0
Diacetyl	5.00	4.96	99.2
Methylglyoxal	0.80	0.82	101.1
Acetol	2.00	2.03	101.5
Diacetyl	0.20	0.19	95.0
Methylglyoxal	5.00	4.92	98.4

Distillation of glucose, fructose, and dihydroxyacetone. One hundred ml of an appropriate phosphate buffer solution of the desired pH was added to a 250 ml distillation flask in a heating mantle. The buffer was brought to boiling and an amount of either glucose (3.60 g), fructose (3.60 g), or dihydroxyacetone (1.80 g) to yield a 0.2 M solution was added. The buffered sugar solution was distilled and the first 2 ml were discarded. A 25 ml volumetric flask in an ice water batch was then used as the receiver and 25.0 ml of the distillate collected in approximately 8 min.

The 25 ml of distillate from each of the buffered sugar solutions was quantitatively transferred to the bisulfite ion exchange column and drained to the top of the resin. Five hundredths molar sodium chloride was then added to the column to elute the carbonyls. The effluent was collected in 10 ml portions and the fractions containing the acetol, diacetyl, and methylglyoxal were identified, combined and analyzed as previously described.

RESULTS AND DISCUSSION

By means of the bisulfite ion exchange column it was possible to separate from a mixture acetol, diacetyl, and methylglyoxal, as shown in Table 1.

Since acetol is a ketone which in general forms less stable bisulfite addition products, it is easily eluted from the column by sodium cloride. Diacetyl, a diketone, is more strongly bound to the bisulfite than is the acetol but less than the methylglyoxal which contains both an aldehyde and a ketone group. Thus, using sodium chloride as an eluting agent, acetol is the first to be removed followed by diacetyl and then by methylglyoxal. Table 1 also shows that mixtures of the 3 carbonyls can be separated quantitatively as well as qualitatively. Varying the relative amounts of diacetyl and methylglyoxal to acetol had little or no effect on the amount of recovery of these from their mixture. Further, these data demonstrate that the bisulfite ion exchange procedure is applicable to the analysis of sugar solutions (distillate) for acetol, diacetyl, and methylglyoxal.

Solutions of 3 sugars—glucose, fructose and dihydroxyacetone—each buffered at a different pH to cover the range of pH 4 to 11.8, were distilled and from the distillate acetol, diacetyl and methylglyoxal were separated and

determined. The concentration of these carbonyls in 25 ml of the distillate are plotted as the concentration versus pH of the sugar solutions to obtain the curves given in Figures 1, 2, and 3. The concentrations of the carbonyls are expressed logarithmically so that the large differences in amounts of the compounds produced can be shown.

Figure 1 shows that methylglyoxal is produced from solutions of dihydroxy-acetone buffered at pH 4 to 9 with the concentration of methylglyoxal increasing rapidly as the pH approaches 4. Likewise, Figure 1 shows that the amounts of both acetol and diacetyl produced from dihydroxyacetone buffered

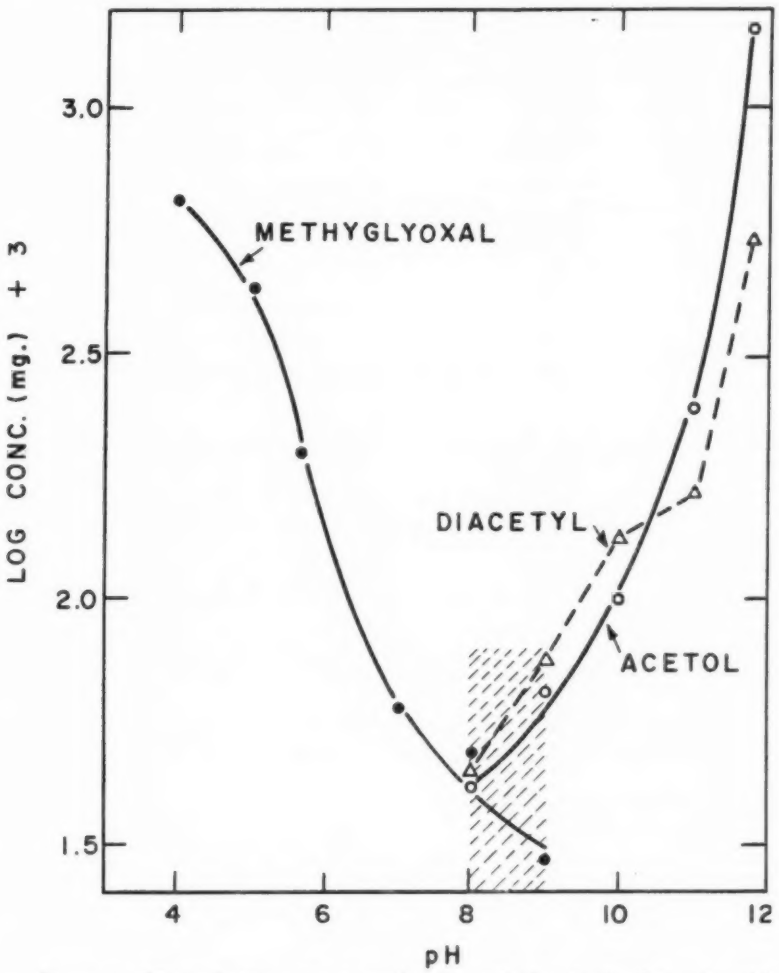
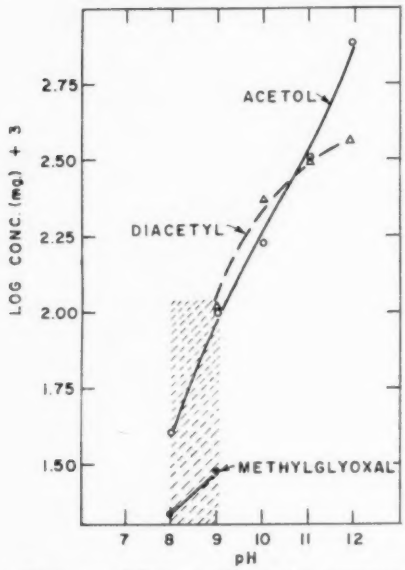


Figure 1. Effect of pH on the formation of methyglyoxal, diacetyl and acetol from dihydroxyacetone.



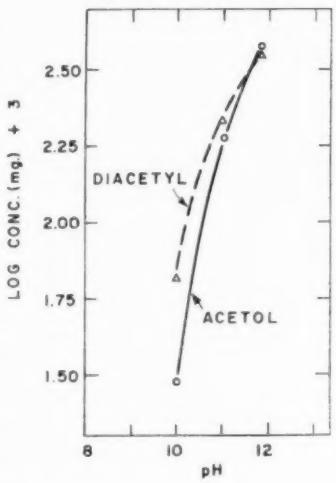


Figure 2. Effect of pH on the formation of methylglyoxal, diacetyl and acetol from fructose.

Figure 3. Effect of pH on the formation of methylglyoxal, diacetyl and acetol from glucose.

at pH 8 to 11.8 increases rapidly as the pH becomes more alkaline. The figure also shows that no detectable amounts (1.0 mg/liter) of acetol or diacetyl were found below pH 8, and above pH 9 no methylglyoxal was found.

Acetol and diacetyl are present in the distillates from alkaline solutions of fructose and glucose, as shown in Figures 2 and 3. Distillates from acid solutions of these 2 sugars yield no detectable amounts of methylglyoxal (0.8 mg/liter). Since it has been shown in Figure 1 that the distillates of acid solutions of dihydroxyacetone contain methylglyoxal, its absence from the distillates of glucose and fructose presents indirect evidence that heated acid solutions of either of these sugars do not favor the production of dihydroxyactone.

The shaded areas in Figures 1 and 2 indicate a pH range (8–9) in which all three of the carbonyls, acetol, diacetyl and methylglyoxal, are present in the distillates of either dihydroxyacetone or fructose. The lesser reactivity of glucose is demonstrated since these compounds were not detected in the distillate of this sugar in this pH range (Figure 3).

Although the amounts of acetol, diacetyl, and methylglyoxal (0.8 mg — 3 mg/liter) determined in the distillates from mild alkaline solution (pH 8–9) of fructose and dihydroxyacetone indicate that the production of these compounds is not favored, with more alkaline conditions relatively larger quantities of acetol and diaceyl are formed (Figures 1, 2), and with more acid conditions relatively more methylglyoxal is produced (Figure 1). Thus, the variation in both the distillate products and in the amounts of these products formed

emphasizes the effect which pH has on the production of acetol, diacetyl, and methylglyoxal from the reducing sugars. This effect is probably related to enolization, rearrangement, or fragmentation of the sugars caused by the pH of the solution.

CONCLUSIONS

By means of a bisulfite ion exchange procedure for the quantitative separation of acetol, methylglyoxal and diacetyl it has been possible to show that the factors which influence their formation are related to the particular reducing sugar involved and to the pH of the solution.

Increasing acidity favored the production of methylyglyoxal from dihydroxyacetone, with little or no acetol or diacetyl being formed from fructose, glucose, and dihydroxyacetone in acid solution. In strongly alkaline solutions (pH 10–12) acetol and diacetyl were produced from the three sugars. In mildly alkaline solutions (pH 8–9) relatively smaller amounts of acetol, diacetyl, and methylglyoxal were formed from both fructose and dihydroxyactone but not from glucose.

The production or non-production of the carbonyls, acetol, diacetyl, and methylglyoxal from these three sugars suggest that their formation is related to the ease with which heated solutions of sugars enolize, fragment or rearrange under different conditions of pH.

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BROWNING OF SUGAR SOLUTIONS. V. EFFECT OF PH ON THE BROWNING OF TRIOSES[a,b]

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The primary decomposition products of the reducing hexose sugars are generally considered to be glyceraldehyde and its ketoisomer dihydroxyacetone. These two trioses can enolize in alkaline solution to give the 1,2 triose endiol, and it is through this common product that these two compounds are inter-related.

Many investigators *(1, 6, 7, 11, 12, 13, 16, 17)* have shown that acetol, methylglyoxal and triose-reductone are produced from glyceraldehyde, dihydroxyacetone and from the hexose sugars. Sattler and Zerban *(14)* have suggested that glyceraldehyde and dihydroxyacetone are intermediates in the formation of acetol methylglyoxal and triose-reductone. Likewise it has been suggested by Zerban and Sattler *(18, 19)* and by Enders *(5)* that methylglyoxal, acetol and triose-reductone have a role in the darkening (browning) of hexose and triose sugars. It would appear that the pH of sugar solutions has a pronounced effect on the production of these carbonyls. It has been shown by Meyerhoff and Lohmann *(11)* and Thornton and Speck *(16)* that in acid solution methylglyoxal is formed from dihydroxyacetone and by Nodzu *et al (11)* and Prey *et al (13)* that acetol, and by Bauer and Teed *(2)* and Weygand *(17)* that triose-reductone are formed in alkaline solutions. These findings together with work done at this laboratory *(8)* suggest that browning (caramelization) of hexose and triose sugars proceed through the intermediate formation of methylglyoxal, acetol and triose-reductone depending upon the pH of the solution.

This study was undertaken to obtain a better understanding of the intermediates formed in the browning of the trioses, dihydroxyacetone and glyceraldehyde, in solutions of pH 4 to 11.

EXPERIMENTAL

Materials. *Triose reductone:* Triose reductone was obtained from Wallerstein Laboratories as a dark brown amorphorous material which was purified by sublimation under vacuum (2 mm) at 40° C. The white sublimate was stored under nitrogen.

Acetol: Acetol was obtained from Bios Laboratory and purified by vacuum distillation. The fraction boiling at 45° C (10 mm pressure) was collected and stored over Drierite.

Methylglyoxal: Methylglyoxal was obtained from Bios Laboratory as a 30% aqueous solution. This was extracted with 2 portions of diethyl ether. The ether extracts were combined and the ether evaporated at reduced pressure. The resulting liquid distilled at 50° C (15 mm). The neutral straw-colored distillate was dissolved in 3 volumes of water and stored at 4° C.

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[a] Presented at the 136th National Meeting of the American Chemical Society, Atlantic City, New Jersey, September 13-18, 1959.

[b] Based on an investigation reported in a thesis submitted by H. G. Lento to the Graduate School of Georgetown University as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[c] Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia 18, Pennsylvania.

Glyceraldehyde: This was obtained from Nutritional Biochemicals.

Dihydroxyacetone: This was obtained from Nutritional Biochemicals.

The purity of the triose-reductone sublimate was 99.4% determined titrimetrically with 2,6-dichlorophenolindolphenol *(9)*.

The concentration of the acetol and methylglyoxal solutions and the purity of the glyceraldehyde and the dihydroxyacetone were determined by periodic oxidation *(4)* and as the 2,4-dinitrophenylosazones. The concentration of the acetol and the methylglyoxal solutions were 85.2% and 35.5% respectively. The purity of the glyceraldehyde and that of the dihydroxyacetone was 99.3% and 99.5% pure, respectively.

Two-tenths molar equeous solutions of each of the 5 carbonyls were prepared and constituted the stock solutions.

Buffers: The phosphate buffer solutions were 0.25 molar disodium acid phosphates adjusted to the desired pH with either one normal sodium hydroxide or with 85% phosphoric acid.

Procedure. Due to the reactivity of the carbonyl compounds with oxygen, care was taken to displace the air completely from all containers with nitrogen. The water used was first boiled to degas it and then swept with nitrogen. All solutions were stored under nitrogen.

The browning reactions were carried out in the reaction vessel and sampler shown in Figure 1.

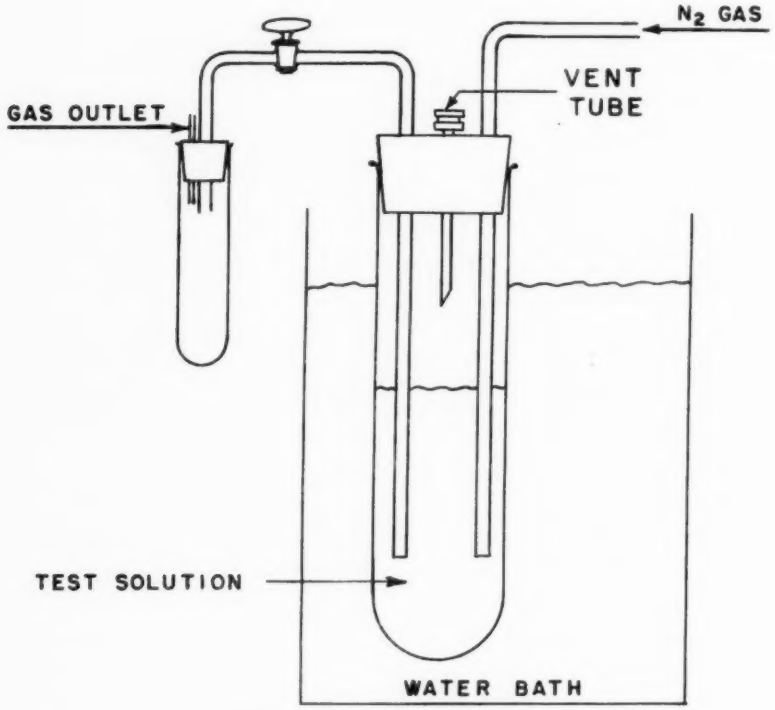

Figure 1. Sampling apparatus.

With the rubber stopper removed, 90 ml of the appropriate buffer solution was placed in the large tube (reaction vessel) and the tube lowered into a 100° C water bath to a depth to cover the buffer in the tube. The stopper was replaced and nitrogen swept through the system to free it from oxygen. Ten ml of the 0.2M stock solution of the particular carbonyl was then added to the hot buffer solution by partially removing the stopper and inserting the tip of the pipet. The stopper was tightly seated and samples were withdrawn at different times by closing the vent tube with a finger tip thus causing the nitrogen gas to force the carbonyl buffered solution, approximately 10 ml, over into the sample tube which was kept chilled in an ice water bath. The sample tube was then disconnected from the system, stoppered and the spectral transmission of the carbonyl-buffer solution measured at 450 mmc using a Coleman Spectrophotometer.

RESULTS AND DISCUSSION

The rate at which color was produced at a given pH by each of the carbonyls was determined by measuring the absorbance of the carbonyl-buffered solutions heated at 100° C for different lengths of time. Since the rate of color formation in the acid solutions was slower, samples were heated for longer periods of time. Since all solutions were adjusted to contain 0.02M of the respective carbonyl the rates and amounts of color produced were characterized by plotting the absorbances of each of the solutions versus heating times to obtain the curves shown in Figures 2 and 3.

Triose reductone has been implicated in the alkaline degradation of reducing sugars and their caramelization reaction products *(18, 19)*. These studies have shown that heated, deoxygenated solutions of reductone produced no color over the pH range of 4 to 11. This is concordant with observations regarding other reductones in which the dehydroforms were the ones which browned *(3)*.

The greatest amount of color was produced by the other 4 carbonyls at the highest pH with solutions of methylglyoxal browning fastest and dihydroxyacetone the least.

The slowest rate of browning for the 4 carbonyls was in the acid range. At pH 4 and 5 the amounts of color produced by glyceraldehyde and dihydroxyacetone were so small (0.02 absorbance even after 75 min at 100° C) that rates of their browning were not plotted. However, when these solutions were heated for much longer periods of time (2 hr) it was shown that the rate of color production was linear.

Since all of the curves, Figures 2 and 3, are linear their slopes were taken as a measure of reaction rates. From these were obtained reaction constants. These reaction constants were then plotted as a function of pH to give the curves in Figure 4.

The slope of these curves, rate constants of color production versus pH, show that the curve for acetol is steepest, indicating the most rapid change in color with pH increase. The curve for methylglyoxal has the least slope, indicating the lowest increase in reactivity with increasing pH. The curves for dihydroxyacetone and glyceraldehyde are identical and superimposed. The slope of these curves indicates that the rate constants (color formation) is not a straight line function. It is significant that a change in the dihydroxyacetone-glyceraldehyde curves occurs in the pH region of 8–9. Above pH 9 and below pH 8 the slopes of the curve are quite different, indicating that changes are occurring in both the dihydroxyacetone and glyceraldehyde solutions as the pH of the solutions is changed.

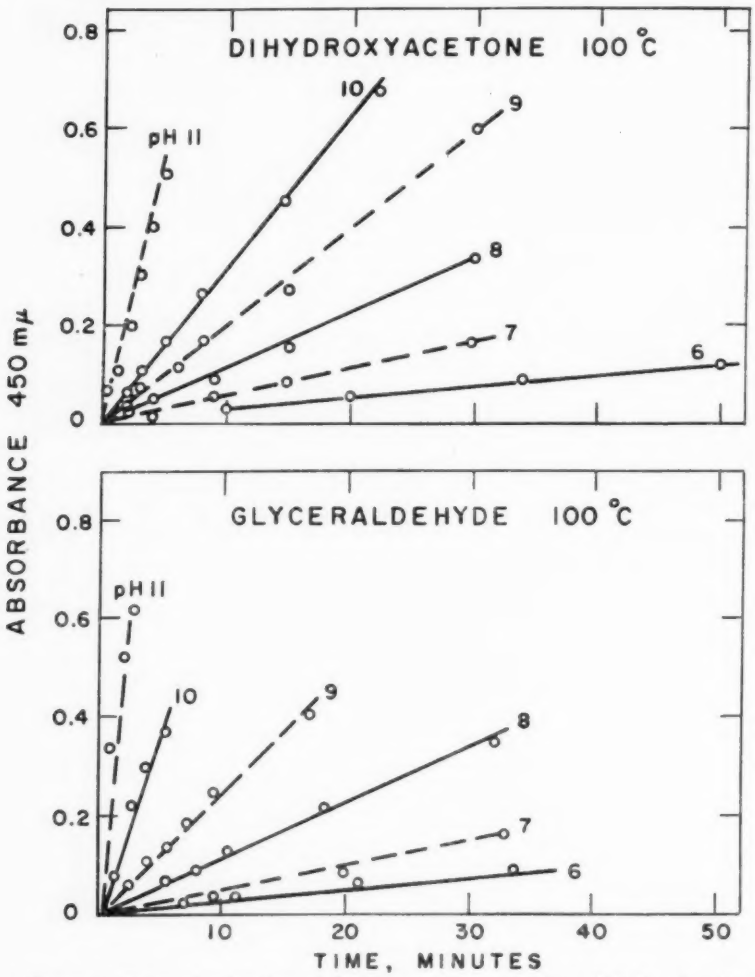

Figure 2. Rates of coloration of dihydroxyacetone and glyceraldehyde at various pH values.

That portion of the curve between pH 4 and pH 8 is similar to, but less steep than, that for methylglyoxal except for that portion close to pH 4. This strongly suggests that the formation of color is due, at least in part, to the formation of some constituent from both dihydroxyacetone and from glyceraldehyde. This formed constituent is no doubt methylglyoxal since it has been shown by earlier work in this laboratory *(8)* that the formation of methylglyoxal from these two triose sugars is increasingly favored as the pH is decreased to 4. Previous work here *(8)* has also shown that as the pH of

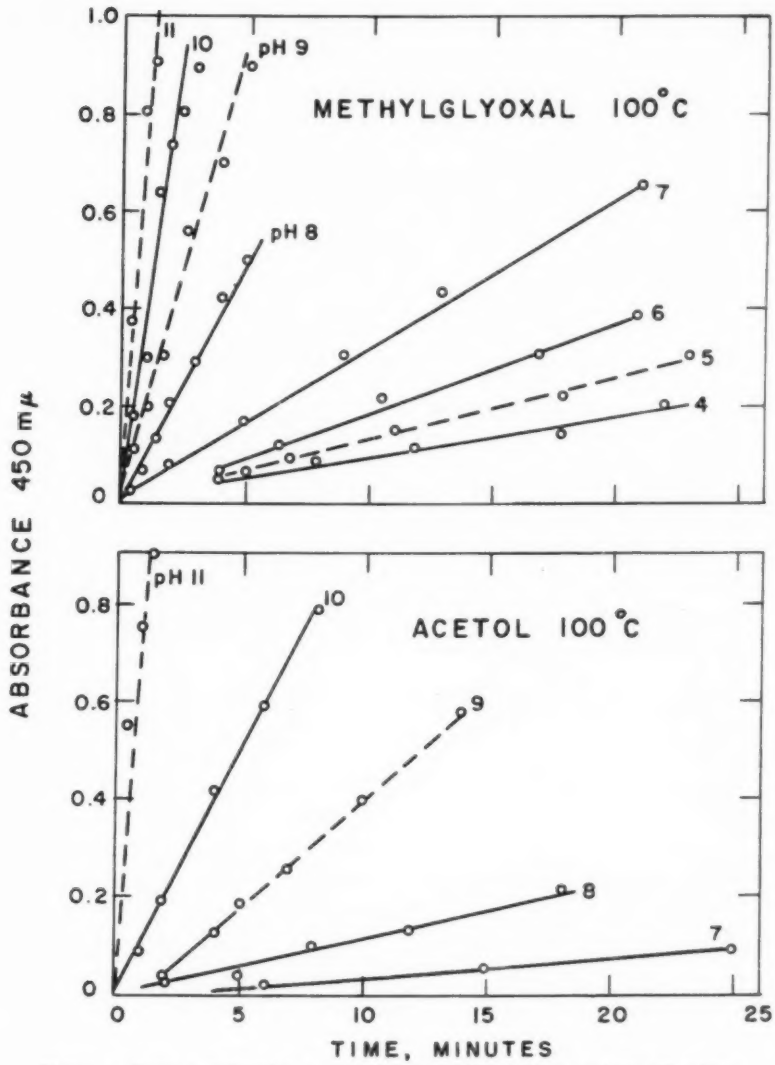

Figure 3. Rates of coloration of methylglyoxal and acetol at various pH values.

solutions of these two sugars is decreased below 7 little or no acetol is formed. This indicates that methylglyoxal is being formed and that it is involved in the acid browning of dihydroxyacetone and of glyceraldehyde.

Above the inflection point of the curve, pH 8–9, the change in the coefficient rate of browning with pH again suggests the formation of a constituent from the two triose sugars, which is not methylglyoxal but very probably acetol.

Previous work has shown *(8)* that as solutions of these two sugars become more alkaline the formation of acetol is favored. With conditions favorable for the production of acetol coupled with the fact that alkaline solutions of acetol brown rapidly, the activation (browning) curve becomes steeper as pH 11 is approached.

It is, therefore, assumed that the successive reactions which bring about color formation in heated solutions of dihydroxyacetone and glyceraldehyde is due to the formation of methylglyoxal and/or acetol whose production is dependent upon the pH of the solution.

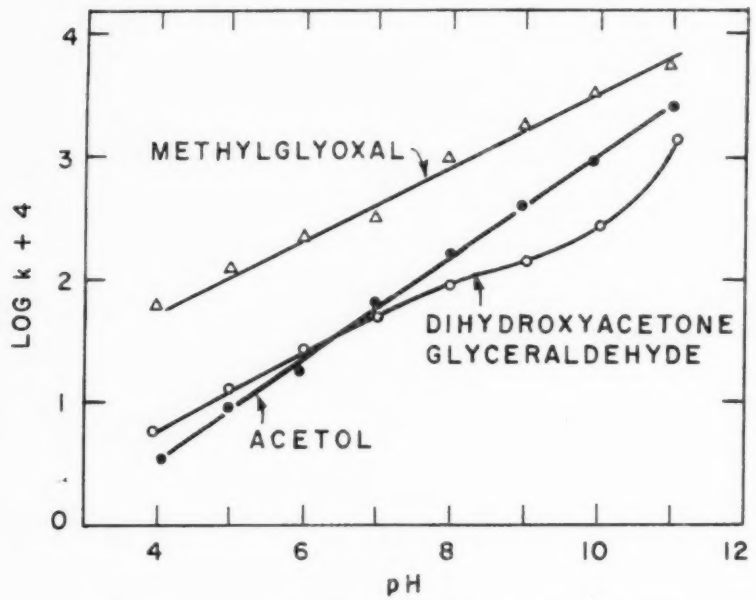

Figure 4. Relationship between pH and log k for rate of coloration.

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VOLATILE CARBONYL COMPOUNDS OF COOKED CHICKEN. II. COMPOUNDS VOLATILIZED WITH STEAM DURING COOKING

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(Manuscipt received October 26, 1959)

Flavor improvement and prevention or postponement of appearance of off flavor in poultry products is of primary importance to the poultry industry. A rational approach to these problems requires a thorough understanding of the flavor characteristics of the products. As a step in this direction, investigations of the fundamental nature of poultry flavor have been under way at this laboratory.

It was recently established *(8)* that a considerable amount and variety of volatile carbonyl compounds may be isolated from chicken cooked in water under atypical oxidation-favoring conditions. This work was extended to include a study of the approximate amount and type of steam-volatile carbonyl compounds which may be isolated from chicken cooked in water under more normal conditions. In the present study, raw cut-up chicken and water were brought to boiling and steam which evolved was condensed and passed into 2,4-dinitrophenylhydrazine reagent. Separation of the mixture of hydrazones obtained into components which permitted unequivocal or tentative identification of many of the carbonyl compounds is described. The significance of these findings to chicken flavor is discussed.

EXPERIMENTAL

Chickens. Hens, culled from laying flocks, were processed to the ready-to-cook point at a commercial processing plant. They were obtained shortly after coming off the processing line, were transported to the laboratory packed in crushed ice, and promptly transferred to individual polyethylene bags, frozen, and held at −30° F until used.

Isolation of steam volatile carbonyl compounds from chicken. Essential features of the apparatus used for isolation of carbonyl compounds as 2,4-dinitrophenylhydrazones are shown in Figure 1. The combination cooking and distillation pot (A) measuring 12 inches in diameter and 13 inches high, was of stainless steel. The lid was modified by providing it with a stainless steel outlet tube and a neoprene gasket that assured a lid-to-pot seal when the lid was held in place with "C" clamps. A stainless steel rack was provided to keep the chicken off the bottom of the kettle to avoid the possibility of burning the chicken by local overheating. The isolation procedure was a batchwise operation in which the cut-up carcasses of five birds (average weight, 5.6 kg) were placed on the rack. Water, 1.07 times the weight of the chicken, was added. The lid was clamped in place and its outlet tube connected to the condenser and traps as shown. The pot was then heated with a gas burner and after gentle boiling started, this state was continued for 4 hr. Steam escaping from the pot was condensed and passed into the 2,4-dinitrophenylhydrazine reagent in trap B. Low boiling carbonyls which might escape this trap were captured by a second trap, C. During the 4 hr cooking, about 4 liters of water distilled. Following a run, contents of the traps were permitted to stand 2 days. Then the hydrazones were filtered off through a fine

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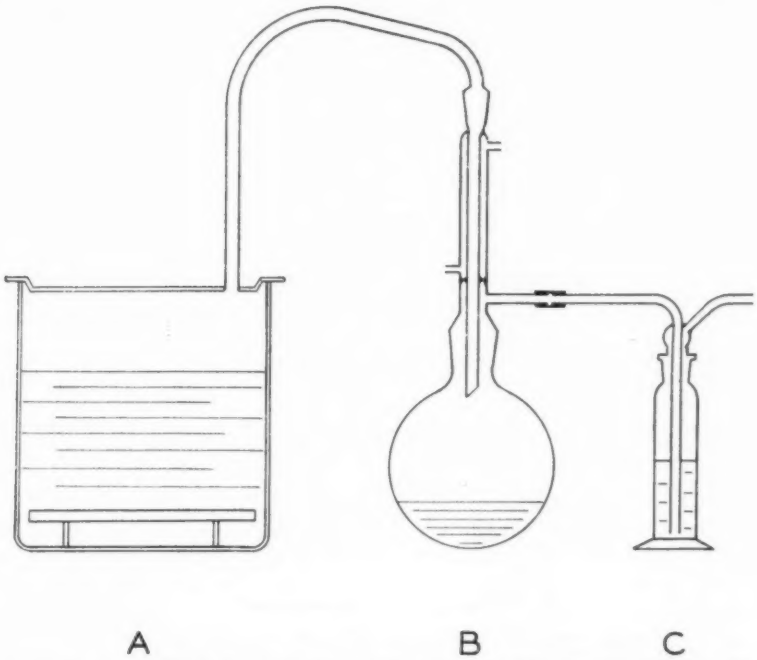

Figure 1. Apparatus used to isolate steam volatile carbonyl compounds from chicken. A—Stainless-steel kettle containing chicken and water heated to boiling. B—5-liter flask containing 500 ml of 2,4-dinitrophenylhydrazine solution (2 g/liter in 2 N HCl). C—Trap containing 200 ml of 2,4-dinitrophenylhydrazine solution (2 g/liter in 2 N HCl).

sintered glass filter. Hydrazones obtained from each trap and from each batch were combined to give a total of 651 mg of 2,4-dinitrophenylhydrazones from 50 birds (56 kg).

Fractionation and identification of 2,4-dinitrophenylhydrazones. A rough preliminary fractionation of the hydrazones was accomplished by taking advantage of solubility differences as previously described *(8)*. The "polycarbonyl" fraction was recrystallized 3 times from nitrobenzene before characterization. The nature of hydrazones, other than the material that crystallized from nitrobenzene, which may have been present in this fraction, was not investigated. The monocarbonyl fraction was divided into approximately 3 equal portions and each was chromatographed on a silicic acid-Celite column as previously described *(8, 9)*. Corresponding preliminary fractions so obtained were combined and rechromatographed on silicic acid-Celite columns until no further separation occurred or the size of the fraction became so small that it was not profitable to continue with this technique. At this point, those fractions which were of sufficient size and of reasonable purity were recrystallized one or more times from ethyl alcohol and characterized by the methods shown in Table 1. Those fractions which were too small to permit unequivocal characterization and which could be further separated by paper chromatography were tentatively identified by their migratory behavior and spectral properties on the paper chromatograms. Methods employed in carrying out these operations have been described elsewhere *(5, 8)*. Authentic n-alkanal, n-alk-2-enal, and 2-alkanone 2,4-dinitrophenylhydrazones required were available from previous studies and are described elsewhere *(5, 8, 9)*. Authentic n-alka-2,4-dienals and the 2,4-dinitrophenylhydrazone derivatives required are those described by Pippen and Nonaka *(10)*.

Estimation of carbonyl in chicken broth. Carbonyl in broth was estimated by modifying the methods of Iddles and Jackson *(1)* and Lohman *(3)*. Hydrazone formation was accomplished by mixing 25 ml of broth and 25 ml of 2,4-dinitrophenylhydrazine reagent (a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl) and permitting this solution to stand 1 to 18 hr at room temperature. To prevent emulsion formation it was necessary to deproteinize before extracting the hydrazones. This was accomplished by adding 10 ml of 10% sodium tungstate solution followed by centrifugation. The supernatant was extracted with hexane in a liquid-liquid extractor for 1 hr. The precipitate, after treatment with 5 g of sodium bicarbonate, was extracted 4 times with hot ethanol (55 ml total). The ethanol extract, after addition of 25 ml of water, was extracted in a liquid-liquid extractor for 1 hr. Hexane extracts were combined, anhydrous sodium sulfate added, and the extracts permitted to stand overnight at room temperature. The extract was filtered, diluted to 200 ml and the absorbancy determined at 340 mμ on a Beckman DU spectrophotometer. A blank was run, with water substituted for broth. From the net absorbancy, the concentration of carbonyl in broth was calculated by using the molar absorption coefficient, 22,500, as determined for authentic n-heptanal-2,4-dinitrophenylhydrazone in hexane. n-Heptanal and cyclohexanone, added to chicken-broth samples at concentrations of 1.3×10^{-5} and 1.5×10^{-5} moles per liter, respectively, were recovered by this method at 79 and 93% levels, respectively. Carbonyl values obtained by the method described here are obviously estimates, because carbonyl compounds in chicken broth are known to include those whose 2,4-dinitrophenylhydrazone derivatives show molar absorption coefficients different from that of n-heptanal-dinitrophenylhydrazone. Also some carbonyl 2,4-dinitrophenylhydrazones, such as the bis derivative of diacetyl and possibly others yet unidentified, have very limited solubility in hexane. This consideration, together with less than theoretical recovery of carbonyl added to broth, means that estimates of carbonyl in chicken broth obtained in this study probably represent minimum values.

RESULTS AND DISCUSSION

In confirmation of previous results [Pippen *et al (8)*], it was found that a 2,4-dinitrophenylhydrazone identical to the bis-2,4-dinitrophenylhydrazone of diacetyl and/or acetoin could be crystallized directly from the "polycarbonyl" fraction. The properties of the recrystallized material are listed at the bottom of Table 1. The relatively large amount of this fraction indicates that diacetyl and/or acetoin occurs as one of the most abundant carbonyl compounds.

TABLE 1

Principal carbonyl compounds isolated

Carbonyl compound identified	Yield of crude fraction, mg	Properties of purified 2,4-dinitrophenylhydrazones		
		Melting point, °C	λ max, mμ	Additional methods of characterization
Acetaldehyde	37	166 –168	355.5 [1]	3, 4, 5
n-Hexanal	125	105 –108.5	357.5 [1]	3, 5
C_{16} and C_{18} aldehydes	16	100 –104.5	357.5 [1]	4, 6
n-Deca-2,4-dienal	32	141.5 –144.5	391 [1]	3, 4, 5
Diacetyl and/or acetoin	191	[4]. > 300	392.5 [2]	4, 7

[1] Chloroform solvent.

[2] Benzene solvent. Authentic diacetyl-bis-2,4-dinitrophenylhydrazone in benzene solvent exhibited at a maximum at 392 mμ.

[3] Melting point not depressed by mixing unknown with authentic sample.

[4] The infrared spectrum of the unknown was identical, except for minor differences, with the infrared spectrum of the authentic sample.

[5] The migratory and spectral properties of the unknown on paper chromatography *(5)* were compared to authentic samples.

[6] Paper chromatography *(5)* of this fraction revealed two spots having R_h values of 2.56 and 2.85 and λ max., mmc of 369 and 368 respectively.

[7] The unknown exhibited a blue-violet color in alkaline ethanol.

Table 1 also lists the most abundant monocarbonyl 2,4-dinitrophenylhydrazones which were identified. Carbonyl compounds unequivocally identified include acetaldehyde, n-hexanal, and n-deca-2,4-dienal. The remaining fraction, m.p. 100 to 104.5° C, resisted further efforts to purify it by means of column chromatography and recrystallization. Its rapid movement on column chromatography suggested that it consisted of a derivative of considerable chain length. As indicated in Table 1, the evidence strongly suggests that this fraction consisted of the mixed derivatives of the C_{16} and C_{18} n-alkanals.

It was evident that 2,4-dinitrophenylhydrazones of many carbonyl compounds, in addition to those listed in Table 1, were present to a lesser extent. This was apparent on the one hand by observing that, after no additional hydrazones could be eluted from the column with the petroleum ether-ethyl ether mixture employed, additional bands could be eluted with chloroform. These consisted of only a fraction of a milligram each and gave violet to blue colors in alkaline ethanol, indicating that they were derivatives of dicarbonyl compounds in which the carbonyl functions are on adjacent carbon atoms *(4)*. Since methods for separating homologues of these compounds have not been developed and authentic samples were not available, these fractions were not investigated further.

On the other hand, it was found that some portions of the mono-carbonyl-2,4-dinitrophenylhydrazone fraction could be profitably investigated by a paper chromatography method previously described *(5)*. This method provides both migratory and spectral data, thus enhancing the probability of assigning the correct identification to a component separated on the paper chromatogram as compared to dependence on migratory data alone. However, it is recognized that identities so assigned are not necessarily unequivocal. Table 2 lists migratory and spectral properties and tentative identity of 2,4-dinitrophenylhydrazones separated by paper chromatography from the mono-

TABLE 2

Tentative identity of carbonyl compounds which occurred in minor amounts. Identity is based on the mobility (R_h) of the 2,4-dinitrophenylhydrazone on paper chromatograms and determination of spectral properties of hydrazones directly on the developed chromatogram

R_h[1]	λ max, mμ	Carbonyl compound indicated
0.56	369	n-Butanal
0.68	370	n-Pentanal
1.00	369	n-Heptanal
1.21	370	n-Octanal
0.57	374	Acetone
1.03	373	2-Pentanone
1.40	374	2-Heptanone
0.33	390	2-Butenal
0.62	388	2-Hexenal
0.74	389	2-Heptenal
0.89	389	2-Octenal
1.01	389	2-Nonenal
1.21	389	2-Decenal
0.46	401	2,4 Heptadienal

[1] R_h equals distance moved by n-heptanal-2,4-dinitrophenylhydrazone/distance moved by unknown 2,4-dinitrophenylhydrazone.

carbonyl 2,4-dinitrophenylhydrazone fraction. It may be seen that these include representatives from the n-alkanals, 2-alkenals, 2,4-alkadienals, and 2-alkanones.

It is of interest to compare total carbonyl in chicken broth to the amount recovered in the steam distillate. Analysis of 4 different chicken-broth samples showed carbonyl concentrations ranging from 9.4 to 16.8 x 10^{-5} moles of carbonyl per liter, the average being 14 x 10^{-5}. The total amount of broth prepared, as a minimum estimate, would be 56 liters. Hence, the total amount of carbonyl present would be 784 x 10^{-5} moles, which, in terms of acetaldehyde 2,4-dinitrophenylhydrazone would amount to 1.75 g. Since only 651 mg of 2,4-dinitrophenylhydrazones were isolated in the steam distillate, it is apparent that considerably less than half of the total carbonyl in the broth was recovered in the distillate.

These results establish for the first time that volatile carbonyl compounds can be isolated from fresh, good-quality chicken, cooked in water for a 4-hr period. Qualitatively, carbonyl compounds identified show similarity to those found in a previous study *(8)* in which diacetyl and/or acetoin was found to comprise the major portion of the polycarbonyl fraction, and n-hexanal, acetaldehyde, and a C_{10} to C_{12} 2,4-dienal were major components of the monocarbonyl fraction along with lesser amounts of other aldehydes and ketones. The most striking difference between results reported here and those previously obtained *(8)* is the appearance of the longer chain aldehydes. Their appearance in the present study is attributable to their volatility with steam, whereas in the air entrainment method of isolation *(8)* they were apparently not sufficiently volatile to escape from the chicken-water mixture. Although the long chain derivatives isolated show properties which are indistinguishable from authentic C_{16} and C_{18} n-alkanal 2,4-dinitrophenylhydrazones, the possibility remains that they may contain unsaturation in one or more positions not conjugated with the carbonyl function. This possibility must be kept in mind until authentic samples and methods become available for establishing the presence or absence of isolated double bonds in the chain of the carbonyl moiety of a 2,4-dinitrophenylhydrazone.

It is clear from these results that fresh, good-quality chicken gives rise to a complex volatile carbonyl fraction when cooked in water. The obvious implication is that these carbonyl compounds contribute to flavor. Much remains to be determined before it can be concluded in detail what contribution they make to flavor. Impossible to evaluate at present is the possible flavor significance of secondary products which could arise through polymerization, oxidation, browning reactions, etc., involving the reactive carbonyl compounds.

However, it is possible to make some general observations concerning the flavor significance of these compounds. Authentic samples of all carbonyl compounds identified in this study were obtained or synthesized. None of these had flavor characteristics like that of cooked chicken. This observation, and the complex nature of the carbonyl fraction revealed by this study, suggests that typical chicken flavor consists of a blend of a broad spectrum of substances. No attempt was made to evaluate the flavor characteristics of a blend of carbonyl compounds identified in this study because the qualitative and quantitative picture is still incomplete. It is of interest to note that the concentration of total carbonyl reported above in chicken broth exceeds con-

siderably the concentration reported *(2, 6, 7, 11)* as the threshold level for similar types of compounds in various media. Consequently, it seems plausible that carbonyl compounds in chicken broth make a significant contribution, collectively, and possibly individually, to flavor. Of particular interest is the recent report *(6)* that n-deca-2,4-dienal, one of the principal monocarbonyl compounds identified in this study, has unusually potent and significant flavor characteristics.

SUMMARY

Carbonyl compounds comprising representatives of the n-alkanals, 2-alkenals, 2,4-alkadienals, 2-alkanones, and 1,2-dicarbonyl compounds were identified in the steam distillate of fresh, good quality chicken cooked in water for four hours. The distillate from 56 kg of chicken yielded 651 mg of 2,4-dinitrophenylhydrazones. Principal carbonyl compounds unequivocally identified include diacetyl and/or acetoin, acetaldehyde, n-hexanal, and n-deca-2,4-dienal. The tentative identity of 16 other carbonyl compounds, occurring in lesser amount, was established. Evidence was obtained indicating the presence of additional carbonyl compounds which were not identified. None of the authentic carbonyl compounds identified in this study had flavor characteristics resembling the complete flavor spectrum of cooked chicken. However, the concentration of total carbonyl found in chicken broth is sufficient to make it plausible that one or more carbonyl compounds contribute significantly to the over-all flavor.

Acknowledgment

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RHEOLOGY OF COCOA BUTTER. III. CRYSTALLINE CHANGES DURING STORAGE AT VARIOUS TEMPERATURES

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Earlier *(20)*, it was demonstrated that purified cocoa butter undergoes oxidative polymerization during storage, the amount of polymerization being proportional to the time of storage and to the temperature. The initial union of monomers to form dimers probably involves bonding of two triglyceride molecules through the unsaturated fatty acid radical of each. [About 40% of the fatty acid components of cocoa butter are unsaturated: 38% oleic acid *(1)*, 2% linoleic acid *(1, 8)* and possibly 0.2% linolenic acid *(4)*.] If such bonding occurs, there should be a noticeable alteration of the crystalline forms of cocoa butter since free association of the molecules would be more and more limited. This steric effect could also be expressed in the physical properties of the crystallized fat.

Although the crystalline structure of oxidized fats has not been investigated, the normal fats of cocoa butter have been the subject of various X-ray diffraction studies *(7, 11, 14, 15)*. In agreement with the known polymorphism of triglycerides *(2, 5, 10, 13)*, the fats of cocoa butter experience crystalline transformations which are associated with previous treatments and the ambient temperature.

Generally, the most stable crystalline form of a fat is designated as β, the next most stable as β', and the least stable as α. Some writers *(5, 6, 21)* recognize a fourth, very unstable and non-crystalline (*i.e.*, vitreous or "glassy") form as γ. However, this last form is not universally accepted *(7, 13)*. The melting point of any crystalline form varies directly with the relative stability of that form. Lutton *(13)* has shown that the crystal lattice of each form is characterized by specific interplanar distances (spacings) and by a particular intensity distribution in the X-ray pattern. So-called "short" spacings are said to represent interchain repeating distances. These vary slightly for triglycerides of different composition. The "long" spacings are related directly to the length of the fatty acid chains and to the angle of tilt of these chains from the basal plane of the crystal; these are often quite diagnostic for different triglycerides.

The spacings which are encountered in cocoa butter are found also in its two major components: 2-oleopalmitostearin (POS) and 2-oleodistearin (SOS). The characteristic spacings of synthetic POS and SOS are given in Table 1. There is general, although not detailed, agreement between these spacings and those reported for POS fractionated from cocoa butter *(15)* and for SOS from kokum butter *(11)*, respectively.

MATERIALS AND METHODS

The method of cocoa butter purification has been reported earlier *(19)*. Samples of the purified cocoa butter were held at temperatures of 0°, 17.5°, 30°, 45°, and 60° C for 90 days, and one sample was held at 60° C for 270 days. Samples were covered

TABLE 1

Characteristic interplanar spacings for two synthetic fats of cocoa butter

Å

Spacings	Fat							
	2-oleopalmitostearin [1] (POS)					2-oleodistearin [2] (SOS)		
	β 3	β' 3	β' 2	α-sub 3	α 2	β 3	X 3	α 3
Long			44 vs	40 vs	48 vs			
	30.3 vs	33.1 vs				31.9 vs	36.6 vs	41.3 vs
	21.2 w	22.3 m					24.0 m	
	15.8 w			15.0 vw	15.9 s		14.6 m	16.2 m
	12.7 m	13.4 s	13.7 m			12.9 m		
					9.53 vw		9.15 w	9.9 vw
	7.95 s	8.37 w				8.13 m	7.83 w	
	6.97 m	7.47 w				7.01 w		
Short	5.42 m	5.56 w				5.44 m	5.22 m	
							4.74 s	
	4.58 vs	4.56 m				4.62 vs	4.55 m	
	4.24 vw	4.36 m	4.30 m				4.23 w	
	4.05 m	4.11 s	4.08 s	4.1 s	4.16 vs	4.01 m		4.19 vs
	3.86 m	3.84 s	3.77 m			3.86 m	3.88 vs	
	3.72 s	3.68 vw		3.7 m		3.78 w	3.60 m	

[1] Lutton, E. S. in *J. Amer. Chemists' Soc.*, **73**, 5595-5598 (1951).
[2] Lutton, E. S. in *J. Amer. Chemists' Soc.*, **68**, 676-679 (1946).
Symbols of intensity: vs—very strong, s—strong, m—medium, w—weak, vw—very weak.

but not sealed, and temperature variation amounted to ± 1° C. Following these treatments, all the samples were placed in storage at 0° C.

Since it is known that thermal treatment can affect the crystallizing potentiality of a fat, two types of pretreatment were used before the registration of X-ray diffraction patterns. In the first ("warmed") type, the samples were warmed from 0° to 25° C. They remained at this temperature 7 days before being X-rayed. The second ("cooled") set of patterns was obtained after raising the temperature of the specimens to 30° C for 5 days and then lowering it to 25° C and maintaining it at 25° C for 7 days.

Conditions of X-ray exposure were as follows: copper anode (40 Kv, 15 mA), 19μ Ni filter, 0.25 mm pinhole, and specimen—film distance of 4.0 cm[1]. With beam stops up to 3.0 mm in diameter, not all the possible long spacings were obtained. However, all spacings up to 33 Å were clearly defined. By eccentric placement of the beam stop, spacings up to 55 Å could be registered in part.

RESULTS

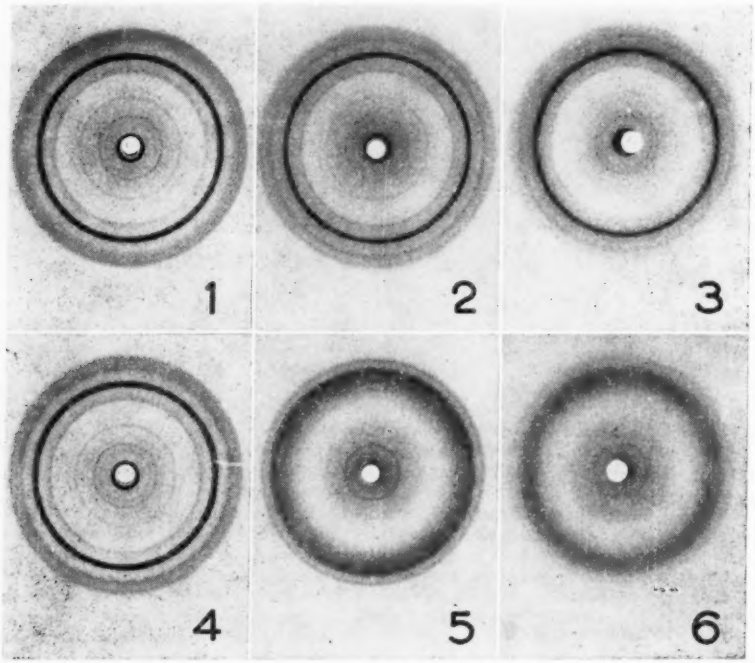
During warming of the samples to 25° C, all began to melt. The amount of melting was least in the 0° C sample (involving principally the surface), and recrystallization was rapid. The 17.5° C specimen experienced more melting and somewhat slower recrystallization. These developments progressed to their maximum extent in the 45° C specimen, which liquefied completely, became translucent, and recrystallized very slowly.

The samples held at 60° C also showed conspicuous melting. However, these did not liquefy completely, nor did they recrystallize in the same way as the other specimens. Recrystallization in the samples held at 45° C and below involved a great increase in opacity and whiteness and a disappearance of surface wetness. On the other hand, even after becoming firm, the 60° C samples remained translucent, and their surfaces appeared wet. (These had been white and very opaque at 0° C). The 60° C specimen held for 270 days was firm sooner than that held for 90 days.

[1] Use of the X-ray diffraction equipment was obtained through the kindness of the U. S. Public Health Service, for which the writer is most grateful.

When the temperature was raised (after solidification) from 25° to 30° C, the 0° C specimen was not visibly changed, the 17.5° C specimen became wet on the surface but remained opaque, the 30° C specimen was a more or less turbid liquid, and the 45° C specimen was a clear liquid. Among the 60° C samples, the 90-day specimen was a wet, translucent gel, but the 270-day specimen showed no sign of melting. On being cooled to 25° C, all specimens became solid, the 45° C specimen being last to crystallize.

Principal X-ray diffraction spacings and their intensities are given in Table 2



Figures 1-6. X-ray diffraction patterns of cocoa butter held under different storage conditions and with warming or cooling pretreatments (see text). Conditions of X-ray: copper anode, 19 μ Ni filter, 4.0 mm specimen-film distance, 0.25 mm pinhole, 25° C sample temperature. Fig. 1. Sample at 0° C for 90 days. Cooled. Fig. 2. Sample at 30° C for 90 days. Cooled. Fig. 3. Sample at 45° C for 90 days. Warmed. Fig. 4. Sample at 0° C for 90 days. Warmed. Fig. 5. Sample at 60° C for 90 days. Cooled. Fig. 6. Sample at 60° C for 270 days. Cooled.

(also see figures 1-6). The spacings of the 0° C sample (in both pre-treatments) may be found among the β forms of both POS and SOS (Figs. 1, 4). With higher temperatures of storage, some of these spacings (*e.g.*, 3.7, 4.0, and 7.1 Å) are less and less intense (Figs. 2, 3), finally disappearing in the 60° C samples (Figs. 5, 6). Most striking is the abrupt transformation of pattern which seems to occur at 60° C (*cf.* Figs. 1-4 vs 5, 6). Note the loss of the spacings at 4.6 and 32.5 Å, the particular increase in intensity of the 4.25 and 15.4 Å spacings, and the appearance of the new spacings at 3.9, 26, and 44.1 Å—in a comparison of the pattern of the lower temperatures with that at 60° C. Note also that generally the rings of the crystalline pattern at 60° C are much weaker after 270 days than earlier, at 90 days (Figs. 5, 6).

The sample at 45° C (Fig. 3), although appearing to present principally the β

TABLE 2

Interplanar spacings of purified cocoa butter stored for 90 days at different temperatures

A

Spacings	Temperature of storage (C°)											
	0		17.5		30		45		60		60 [1]	
	w [2]	c [3]	w	c	w	c	w	c	w	c	w	c
Long							44.1 s	44.1 vs	44.1 vs	44.1 vs	44.1 vs	44.1 vs
	32.5 vs	32.5 vs	32.5 vs	32.5 vs	33.2 vs	32.5 vs	33.2 vs	32.5 vs				
									25.2 m	25.7 m	26.3 m	26.3 m
	16.1 m	16.1 m	16.2 w	15.9 w	16.1 m	16.1 w	15.9 w	15.4 w	15.3 m	15.1 s	15.4 w	15.7 m
	13.1 m	13.1 m	13.1 m	13.1 m	13.1 m	13.1 w	13.1 w	13.1 w				
	8.15 m	8.15 m	8.11 m	7.95 m	8.11 m	8.11 w	8.11 w	8.11 w				
	7.13 m	7.04 w	7.21 w	7.05 w	7.21 w	7.04 w	.....	.....				
Short	5.48 m	5.52 m	5.48 m	5.48 m	5.48 m	5.43 w	5.45 w	5.48 w				
	4.63 vs	4.60 vs	4.61 vs	4.60 vs	4.61 vs	4.57 vs	4.61 vs	4.60 s				4.64 w
		4.30 w		4.27 w		4.27 w	4.25 w	4.25 m	4.28 s	4.24 s	4.25 m	4.23 m
	4.02 s	4.07 w	4.02 s	4.03 m	4.02 m	3.98 m	4.02 w	3.98 m				
		3.90 m							3.90 s	3.88 s	3.89 m	3.85 m
	3.70 s	3.70 m	3.70 s	3.69 m	3.70 m	3.70 m	3.70 w	3.71 w				

[1] Stored for 270 days.
[2] Warmed from 0° to 25° C.
[3] Cooled from 30° to 25° C.
Symbols of intensity: See Table 1.

pattern of the 0° C sample, has crystalline elements of the 60° C samples also. The spacing of 44.1 Å makes its first appearance here. The relatively weaker intensities of the 44.1 and 15.4 Å spacings, in comparison with those of 60° C, indicate that the β crystal and the crystal of 60° C exist side by side in this sample and that there is no transitional crystalline type involved.

Some differences in crystalline organization occur between the two sets of pre-treatments (Figs. 1, 4; Table 2). These differences are seen primarily in the short spacings, which generally are more numerous in the cooling pre-treatment. Attention may be directed to the spacing of 4.07 Å in the 0° C specimen (cooled). This is the only dimension that is related to differences in storage temperature. In the cooled series, it decreases continually to 3.98 Å at 45° C. It cannot be assumed that the line at 3.88 Å, in the 60° C treatment, is the same crystallographic lattice spacing. In the warmed series, a strong line appears at 4.02 Å in the 0° C specimen. This spacing remains unchanged to 45° C, but decreases in intensity as the temperature of treatment is increased.

DISCUSSION

The melting which occurs when the samples are warmed to 25° C from 0° C is not unexpected. Originally, these were put directly into 0° C storage from a molten state. Consequently, they congealed in the α form, and the low holding temperature did not permit any notable polymorphic transformation. Thus, when warmed, they passed through the normal melting points of α forms of POS, 17°–18° C and SOS, 22°–23° C *(11, 14, 15)*. Holding for 5 days at 25° C may or may not bring about a transformation of β' to β. In the case of the present specimens (especially 0°–45° C) the crystalline pattern is already β and not β'. Nevertheless, the introduction of more spacings in the X-ray patterns of the cooled samples shows that the β crystalline fraction is increased by this treatment *(cf. 21)*. This possibly is due to melting and recrystallization of a minority of β' crystals, which are masked by a greater majority of β crystals.

The differences in melting behavior of the samples stored at different temperatures supports a similar observation of Fincke *(8)*, *viz.*, that high temperature treatment (he used 100° C for 2 hr) lowers the melting point of β cocoa butter. Considered together with the decreasing intensity of the crystalline pattern, this phenomenon can certainly be ascribed to the decreasing crystallinity, *i.e.*, greater amorphousness, and probably a larger liquid fraction in the samples following storage at higher temperatures.

Because of their opacity at 0° C and their subsequent melting as the temperature is raised, the 60° C samples may have an α-crystalline form. However, these samples are predominantly gel-like. In addition, the longer the high-temperature treatment, the more highly organized is the gel. This is the anticipated end result of continued polymerization via cross-linking *(9)*.

On the whole, the 60° C samples appear to have some elements of the general β' crystal. The major spacings agree in position, if not in intensity. Thus, oxidation seemingly promotes a "β'" organization in the crystalline lattice. This organizational preference is also suggested in the lowered melting point, the presence of both β and "β'" spacings in the 45° C sample, and the gradual loss of intensity of β spacings. Molecular conformations induced by oxidation therefore tend to prevent β crystallization generally, and permit a pseudo-β' type of lattice to be organized.

In a strict sense, the diffraction pattern of the 60° C samples is neither β' nor β. Perhaps it can be considered as a new, derivative type. There is some resemblance between it and the pattern of the α-sub 3 form of POS

(Table 1) as well as the β' form of other fats. Yet, the appearance of this new pattern, following that of 45° C, is saltatory. If β' or α crystals were being organized in preference to β crystals, this event should be recognizable as a *gradual* consequence of increasing oxidation. Therefore, these crystals appear to be not β' nor α-sub 3 crystals but a new form. Provisionally, they may be designated as "ω" (omega), as an abbreviation for oxidation-derived, crystals.

Lutton *(13)*, following Schoon *(17)*, has depicted the hydrocarbon chains of triglyceride crystals as being arranged at the corners of a rhomb, which latter forms the base of the unit cell. This view is probably correct, inasmuch as (1) strong reflections in crystal lattices of long chain compounds are generally first order spacings of the major, simpler crystallographic planes, and (2) density calculations show that a unit cell so-defined would have approximately the correct density.

Changes in the spacings of the X-ray pattern and in their diffracted intensity indicate changes in crystalline structure. In α, β', and β crystals, the least distance between the fatty acid radicals is far greater than the 1.34 Å length of the C = C bond (which would be the cross-linking bond between two unsaturated fatty acid radicals in a dimer). Thus, it is obvious that the usual α, β' and β crystalline forms cannot be organized in highly oxidized cocoa butter.

Because the oleyl group occupies the 2-position, some sort of tuning fork conformation might be the expected structure of pure or mixed dimers of POS and SOS. Clarkson and Malkin *(5)* and Lutton *(12, 14)* conceive of a unit of this structure in normal fat as consisting of two reversed fat molecules, which lie side by side. Their shanks (oleyl groups) adjoin one another, and their tines (disaturated groups) are opposed. This forms a "triple" chain of an approximately length of 65 Å, whose (020) spacing would be 32.5 Å, as in the β lattice of cocoa butter.

The reversed tuning fork structure described above cannot directly yield a spacing of 44 Å as is found in ω cocoa butter and in some β' and α forms of disaturated triglycerides. Perhaps if the unit cell length were a sextuple chain length (132 Å), 44 Å might be conceived as the (030) spacing. It is more reasonable to consider that instead of being reversed, the paired tuning forks are superimposed, shank to shank and tine to tine. Such a double chain length would then have a first order spacing of 44 Å. A dimer might also be formed from two fat molecules in the "pectinate" configuration (*i.e.*, comb-like arrangement, in which the three fatty acid groups issue unidirectionally from the glycerol group). Thus, a double chain length of about 44 Å would result if, in the long axis of the dimers, glycerol groups adjoined glycerol groups, and the ends of the fatty acid groups adjoined the ends of the fatty acid groups.

Formation of such dimers, in an accelerated manner, could give the marked crystallographic change noted in the 60° C samples. Continued bonding of dimers to monomers or to other dimers eventually decreases the amount of ω crystallinity, yielding a less regular, gel-like structure. Although an initial dimer (and even trimer) structure and eventual gelation are also a consequence of thermal polymerization *(3, 16, 18)*, it has been shown that the contribution of such polymerization here is quite small *(20)*.

SUMMARY

Oxidation of purified cocoa butter results in decreased crystalline organization and increased amorphous fraction, in a direct dependence upon temperature. Potentiality of organization of normal (α, β', β) crystals is progressively decreased, but at some stage in oxidation—probably involving a large amount of dimer formation, the crystalline pattern suddenly changes. It is speculated that a new type of crystal (ω) is produced. This is gradually superseded by a less crystalline, gel-like structure as oxidation continues.

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STUDIES ON TEMPEH—
AN INDONESIAN FERMENTED SOYBEAN FOOD*

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Tempeh, an Indonesian food produced by growing a mold on soybeans (Figure 1) has been suggested as a possible source of low cost protein for child feeding programs in underdeveloped countries *(1)*. Van Veen and Schaefer *(5)* have reviewed the literature and described the Indonesian method of producing tempeh. Their studies suggest that tempeh is more easily digested than the original soybean. Production of tempeh in Indonesia is on a home or small scale basis. The product is not yet adapted to modern commercial methods necessary if tempeh is to play an important part in child feeding programs for the world.

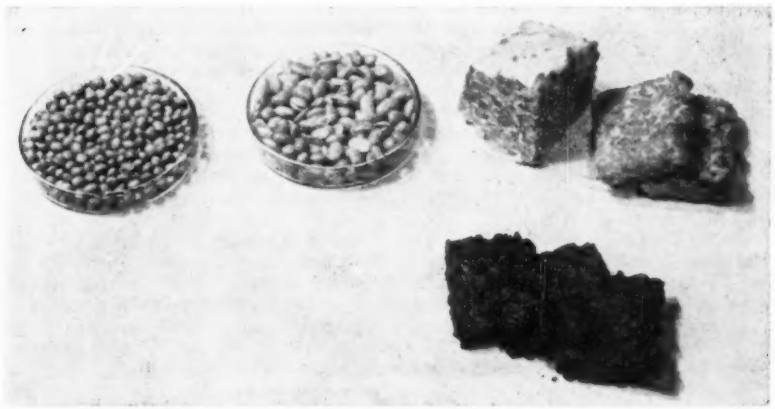

Figure 1. (Back, left to right)—(1) dry soybeans, (2) soaked, peeled, cooked soybeans, (3) raw tempeh bean cake sliced, and (front) deep fat fried sliced tempeh.

It was decided, therefore, to investigate the tempeh fermentation, determine the factors controlling mold growth, and secure the information needed to set up controlled fermentations on a relatively large scale. A study was made also of changes, particularly of soluble soybean solids, occurring during the tempeh fermentation which might support the contention that tempeh was more digestible than soybeans.

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MATERIALS AND METHODS

Essential steps in tempeh production. *Indonesian Method.* The soybeans are hydrated by either soaking overnight in cool water or by soaking for a shorter length of time in hot water. Skins are removed by placing a container of the beans, either before or after cooking, in water and rubbing the beans together with the feet or between the hands. The loosened skins rise to the top of the container and float away with the water. The beans, which are then partially cooked either by steaming or boiling, remain firm. After cooking, all liquid is drained from the beans and they are inoculated with scrapings from a previous fermentation. The beans are then wrapped in large banana leaves and placed in a warm place to ferment. During the fermentation, the beans become bound together into a compact cake by mold mycelia. The individual beans become softened through enzymic digestion. The raw tempeh is sliced and dried or roasted, cooked in soup, or deep fat fried before it is eaten.

Method of production used in present study. The soybeans used in this investigation were medium sized, yellow soybeans of the Seneca variety. Hydration was carried out in batch lots. For every kg of dry beans, 3 liters of water and 30 ml of lactic acid were added. Beans were generally soaked overnight at room temperature.

The acidified soak water was saved and the beans were passed through an abrasive vegetable peeler [b] which loosened the skins. Separation of the skins from the bean cotyledons was accomplished by running water into the vats accompanied by gentle stirring of the beans.

The skinned beans were then returned to the acidified soak water and cooked at 100° C for 90 min. The water was now drained from the beans, which were allowed to cool to about 37° C before inoculation.

The inoculum used was a pure culture of mold isolated from scrapings from a good tempeh fermentation brought here from Indonesia by Miss Yap. The inoculum for large scale experiments was prepared by inoculating the pure mold on a medium consisting of 100 g wheat bran moistened with 300 ml of acidified soybean soak water sterilized in a Fernbach flask for 20 min at 120° C. The cultures were incubated for 7 days at 37° C during which time the culture sporulated heavily. The culture was then lyophilized and ground through a 20-mesh screen in a Wiley mill. One gram of inoculum was added per kg of cooked beans and mixed thoroughly.

The inoculated beans were placed in covered stainless steel trays, in layers 1½ to 2 inches thick. Diffusion of air into the bean mass was facilitated by ⅛ in holes drilled in the top and bottom of the trays at 2-inch intervals. The temperature of incubation was generally 37° C.

During the fermentation, the beans were examined at regular intervals for development of mold, temperature of the fermenting bean mass, sporulation, and production of free NH_3. Samples were removed periodically and analyzed for pH, soluble solids, soluble and total nitrogen, reducing substances, total fiber, and ether-soluble materials.

After the beans were bound together by the mold mycelium, the bean cakes were sliced thin, dipped in 10% salt brine for flavoring, deep fat fried at 190° C until crisp and light brown, and were tested organoleptically.

Soybeans were also sectioned on a microtome before and after mold growth to determine extent of mycelial penetration into the bean and the amount of cellular breakdown. The samples were paraffined, embedded and sectioned at 10 or 12 microns with a Spencer rotary microtome. The sections were stained with thionin, orange G and erythrosin to distinguish mold mycelia from soybean cells *(3)*.

Samples of tempeh were preserved either by quick freezing at −15° C and lyophilization or by dehydration in a circulating hot air oven at 69° C.

ANALYSES

Determination of soluble solids, moisture, pH and nitrogen. A 50-g sample of tempeh was homogenized with 200 ml of distilled water in a Waring blender for 5 min. The pH determination was made directly on the homogenate using a Beckmann Model G meter. The homogenate was then heated in a boiling water bath with constant

[b] Reynolds Electric Company, Chicago.

stirring to raise the temperature to about 90° C in order to destroy the enzymes. This was to prevent enzyme reactions during manipulations, which require several hours. If any delay was required in analysis, the heated homogenates were stored at 1° C. Weighed samples of the heated homogenate were taken for determination of total solids and total nitrogen (macro Kjeldahl). Solids were run by removing the excess water by drying at 70° C for 20 hr. Final drying was accomplished at 70° C for 48 hr in the vacuum oven.

For the soluble solids determination, the homogenate was centrifuged for one-half hour in a Servall centrifuge at 10,600 rpm. The supernatant was then filtered through Whatman No. 1 filter paper to remove free fat which floated on the surface. The supernatant from the Servall centrifuging was then centrifuged for 90 min in a Spinco refrigerated ultracentrifuge at 38,000 rpm. This procedure resulted in a good separation of soluble and insoluble solids. Weighed 5 ml aliquots of the final supernatant were used for determination of soluble solids, soluble nitrogen, and reducing substances as glucose *(4)*.

RESULTS AND DISCUSSION

Microorganisms involved in the tempeh fermentation. The mold isolated from the crude Indonesian tempeh scrapings belonged to the genus *Rhizopus*. It closely resembled the species *Rhizopus oryzae* Went and Prinsen Geerligs *(2)*.

In addition to the mold, a spore-forming *Bacillus* and a non-spore-forming bacterium were present in the crude tempeh scrapings in nearly pure culture for their respective types. No yeasts or other microorganisms were found. However, the dried culture had been stored at room temperature for 2 months and additional microorganisms may have died. Van Veen and Schaefer *(5)* reported that the principal organism involved in the tempeh fermentation was *Rhizopus oryzae*. Although they found numerous bacteria and yeasts present, they concluded that they were not of considerable importance to the process. The present studies have indicated that the bacteria are definitely undesirable, contributing off-odors to the tempeh if they are allowed to develop. If the pH of the beans was adjusted to 5 or below, bacteria did not develop on the beans.

Thus, the mold is the essential microorganism in the tempeh fermentation. Tempeh prepared with the mold, in the absence of other microorganisms was described as "very pure" and "better than tempeh produced in Indonesia" by students from that country.

A number of other cultures of *Rhizopus oryzae*, including NRRL[e] strains 1526, 395, 1528 and 2549, were tried in tempeh fermentation. All strains produced a satisfactory tempeh but required 48 hr to overgrow the soybeans vs 24 hr for our mold at 37° C. Strain No. 2549, which had been isolated originally from a tempeh fermentation and our isolate, which has been assigned number NRRL 2710 by Dr. Hesseltine, differed from the others tested in that they produced enough free NH_3 on ordinary unbuffered laboratory media through proteolytic breakdown to sterilize the culture.

Factors influencing mold growth. In order to be complete, the tempeh fermentation required that two conditions be fulfilled. First, the soybeans had to be bound into a compact cake by the growth of mold mycelium. Second, the soybeans had to undergo a partial digestion by the mold enzymes. Thus, the tempeh fermentation was centered in the mold growth.

It was found that the tempeh mold, *Rhizopus oryzae* would not grow well on the unskinned beans. Even coating the soybeans with ground, roasted

[e] Kindly supplied by Dr. C. W. Hesseltine, U.S.D.A. Northern Utilization and Development Division, Peoria, Illinois.

wheat or other carbohydrate containing nutrients as is successfully practiced in soysauce fermentation did not result in rapid and heavy mold growth. The mold used in the soy-sauce fermentation, of course, belongs to the genus *Aspergillus* and this may explain the different behavior. Thus, skin removal is an essential step by the present process.

Removal of the skins from the hydrated beans was found to be a difficult step, due to the similarity in density of the soybeans and their skins. After the skins were loosened, it was necessary to use a gentle stirring action in the vats to cause the skins to rise slowly in the water. If this was accompanied by a slow flow of water into and out of the upper section of the vat, the skins were gradually removed while the beans settled at the bottom of the vat. The development or adaptation of present decorticating equipment for the removal of soybean skins in the dry state would greatly facilitate large scale production of tempeh.

Oxygen was essential to the growth of the mold. When the layer of fermenting beans was thicker than approximately 2 inches, mold growth became less rapid and less heavy in the center of the bean mass than it was in thinner layers. This limitation of depth imposed the requirement that large scale production of tempeh by present processing methods use a large area of fermenting pans.

It was found that the mold would grow well on beans only when the humidity around the beans was maintained at a high level. The requirements of the mold for both oxygen and high humidity were satisfied by using stainless steel pans with 1/8 inch holes in the tops and bottoms drilled at 2 inch intervals. The holes were sufficient to permit access to air but did not allow enough loss of moisture to interfere with growth of the mold.

Using small ($10 \times 14 \times 2\frac{1}{2}$ inch) pans with loose fitting covers, satisfactory mold growth was obtained without drilling additional holes for access of air as long as the soybean layer did not exceed approximately half the volume of the container.

The tempeh mold grew rapidly and profusely at 37° C. During active mold growth, the temperature of the fermenting beans rose above the incubator temperature. If the temperature was not controlled, it occasionally rose to 49° C at which temperature further mold growth was inhibited. As long as the temperature did not rise above 45° C, mold growth progressed satisfactorily. Temperatures as low as 25° C were used to produce an acceptable tempeh. Such fermentations required as long as 5 days for completion vs 1 day at 37° C.

The pH of soybeans soaked and cooked in water acidified with lactic acid (30 ml acid/3000 ml water/1000 g beans) was about 5. This pH was sufficiently low to inhibit growth of bacteria which would spoil the tempeh but did not interfere with growth of the mold. The mold growth was not inhibited by acidity until the pH fell below 3.5. Then growth was just a little slower.

Flavor of tempeh. Fresh, raw, uncontaminated tempeh was found to have a clean, pleasant odor and a slightly cheeselike flavor. The deep fat fried tempeh had a nutty flavor acceptable to nearly everyone who tested it.

Changes occurring during the tempeh fermentation. Some of the changes which occurred in the soybeans during a typical tempeh fermentation can be followed by referring to Figure 2. The temperature of the fer-

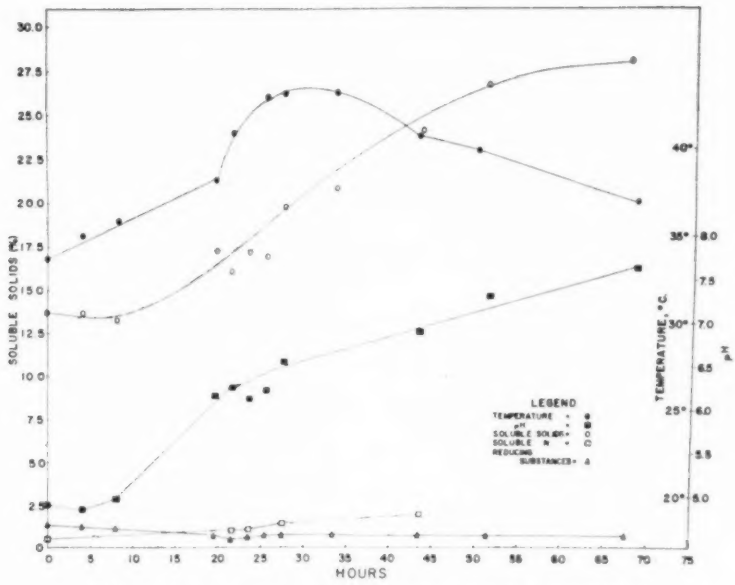


Figure 2. Changes occurring during the tempeh fermentation.

menting bean mass was indicative of the relative rate of mold growth. For the first few hours after inoculation, there was a lag during which germination of spores took place. This was followed by several hours of slow mold growth. When mold growth became rapid, the temperature of the bean mass rose above that of the incubator. After 4 to 5 hr of accelerated mold growth, the temperature reached 43° to 44° C. After the temperature reached its peak, it gradually fell as mold growth subsided. At this stage, the beans were bound into a compact mass by the mycelium. Following rapid mold growth, sporulation and NH_3 production due to protein breakdown appeared. In small quantities neither spoiled the tempeh. Tempeh is quite similar to a Camembert cheese in this regard. Personal preference determines how much digestion the consumer desires, but the majority probably prefer a moderate degree.

During the period of most rapid mold growth, the soluble solids rose from 13 to 21%. Due to further enzyme digestion, the soluble solids continued to rise to 27.5%. At this stage, the sporulation and NH_3 production would be considered too far advanced for many consumers.

The soluble nitrogen rose from 0.5 to nearly 2.0% while the total nitrogen remained relatively constant, about 7.5%.

The pH rose from an initial level of 5.0 to nearly 7.6. It changed from 6.0 to 6.7 during the period of most rapid mold growth. The tempeh was at its optimum quality when the pH was in the range of 6.3 to 6.5.

Reducing substances showed a decline due to their utilization by the mold in its metabolism. They did not regain their initial level even though enzyme breakdown of higher carbohydrates continued.

A special study was made of changes in soybean lipids during the tempeh fermentation, Wagenknecht *et al (6)*. Total fat (as ether soluble solids) remained fairly constant. Over one-third of the neutral fat of the soybeans was hydrolyzed by the mold during a three-day fermentation. Of the free fatty acids liberated, only linolenic acid was utilized by the mold.

There was 3.7% fiber (dry weight basis) in the hydrated, peeled, cooked Seneca soybeans while the finished tempeh contained 5.8% fiber. Tempeh digested soybeans freed of their superficial mold layer showed a reduction in total fiber to 2.8%. The mold mycelium removed from the tempeh contained 7.1% fiber. Thus, it would appear that the mold mycelium is responsible for the increase in fiber content of tempeh above the undigested soybeans.

Results of cytological studies. The results of cytological studies on sections of hydrated, peeled, and cooked soybeans and the beans after fermentation suggest (Figures 3, 4, 5) that most of the mold growth is on the surface of the bean with relatively little penetration of the mycelia either between or into the underlying cells. This suggests that most of the action of the mold upon the beans is by means of secreted enzymes.

In the production of tempeh, the soybeans are only partially cooked and remain nearly as firm as the hydrated beans. After the fermentation the beans are soft and similar in texture to completely cooked soybeans.

Cytological studies were made of hydrated skinned soybeans before and after cooking and after fermentation. Ten-gram samples of the beans or tempeh were beaten with 50 ml of water in Waring blenders for 30 to 180 sec. At 30-sec intervals the suspensions were examined under 100–450X magnification to determine how many intact cells remained. It was found that both the hydrated soybeans and the partially cooked (90 min steam) soybeans contained relatively few intact cells after the 30-sec treatment (Figures 6 and 7). The cells of cooked beans and the tempeh, however, showed a marked resistance to fracture in the Waring blender and remained intact (Figures 8, 9).

The Waring blender produces a violent agitation which would be expected to break up the cells but, surprisingly, the cells of cooked beans and tempeh remained intact. Equally unexpected was the fracturing of cells of hydrated soybeans when they were smeared on a glass slide. The cells of fully cooked beans and tempeh remained intact by this procedure.

Preservation of tempeh. Fresh, raw tempeh remained edible for only a few days when stored at room temperature. Storage stability was achieved by drying. The Indonesians cut the tempeh into thin strips and sun dry it *(5)*. It was found, Table 1, that lyophilization had much less effect upon the pH, per cent reducing substances, and soluble solids including soluble

TABLE 1

Comparative effect of freeze drying versus hot air dehydration (69° C) on several analyses of tempeh

Tempeh	pH	% reducing substances	% soluble solids	% soluble nitrogen
Fresh	6.3	0.71	17.6	2.31
Lyophilized	6.2	0.41	19.5	1.19
Hot air dried	5.3	0.28	13.8	0.61

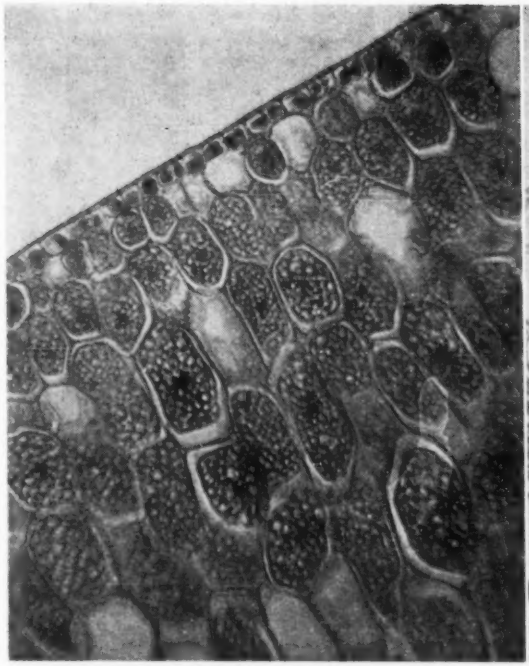


Figure 3. Hydrated soybeans. x 500.

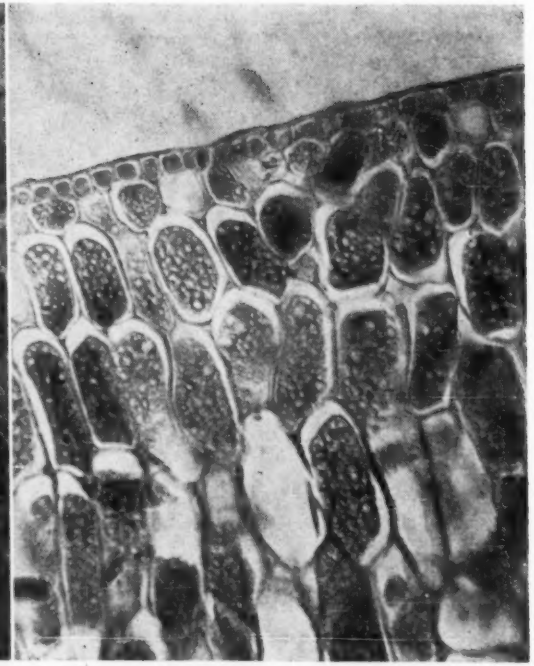
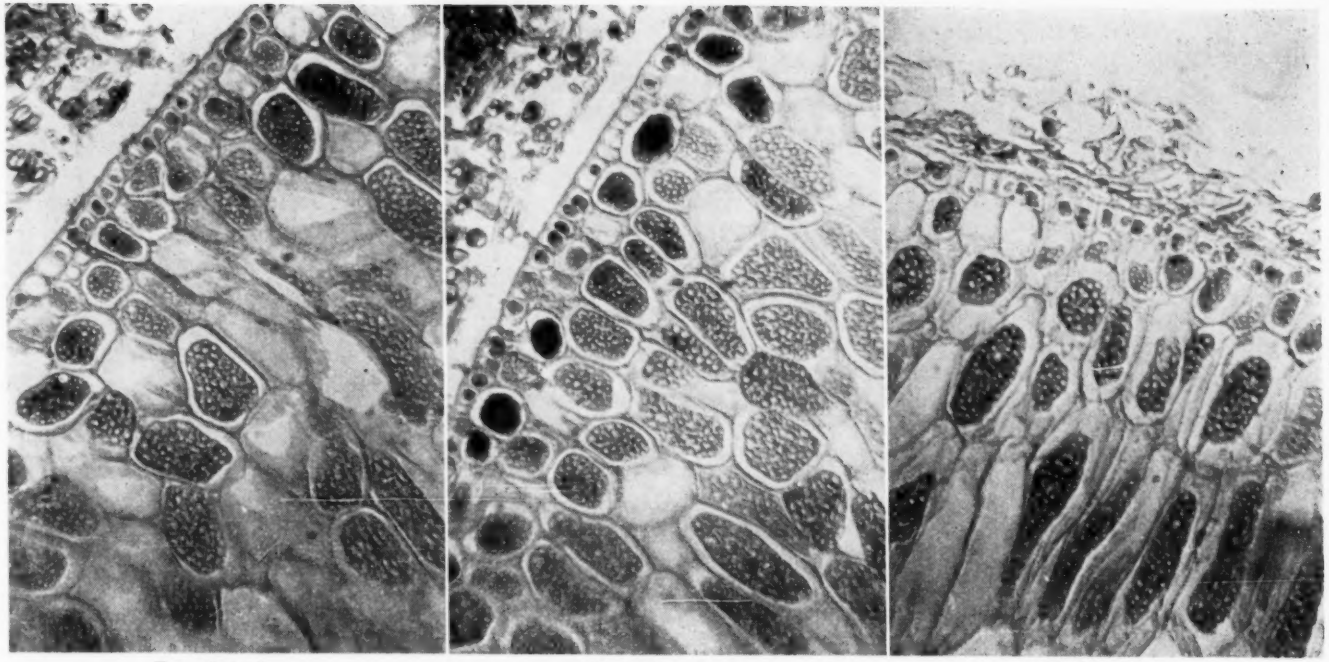


Figure 4. Hydrated steamed soybeans. x 500.



Tempeh 24 hours | Tempeh 24 hours | Tempeh 72 hours

Figure 5. Several sections of tempeh digested soybeans. x 500.

Figure 6. Hydrated soybeans.*

Figure 7. Soybeans steamed 90 minutes.*

* Ten grams beans beaten with 50 ml water in Waring blender 30 sec. All pictures at 200X.

Figure 8. Tempeh.*

Figure 9. Soybeans cooked 1½ hours (120° C).*

* Ten grams beans beaten with 50 ml water in Waring blender 30 sec. All pictures at 200X.

nitrogen, than did dehydration in a circulating hot air oven at 69° C. The reduction in reducing substances can be accounted for in part by the browning reaction which occurs at the higher temperature. However, a substantial reduction in reducing substances occurred even in the freeze-dried product. Whether or not the reductions in solubles represent a serious loss in nutrient value of tempeh has yet to be determined.

Yields of tempeh at various stages of processing. From 100 g raw dry Seneca soybeans (6.8% moisture) were obtained 233 g soaked (62.0% moisture) 202 g skinned (64.0% moisture), 189.5 g cooked (62.4% moisture), and finally 174.3 g of tempeh (61.2% moisture). On a dry solids basis, 93.2 g of raw dry beans produced 88.6 g of soaked beans, 72.7 g of cooked beans, and 67.6 g of tempeh. This represents a recovery of 72.5% of the original dry soybean solids in the form of tempeh. The total losses of solids (27.5%) were distributed as follows: 4.9% leaching during soaking, 17.1% loss during peeling of which 9.6% was due directly to removal of skins, 1.6% leaching during cooking, and 3.9% in the fermentation.

SUMMARY

It was found that the mold *Rhizopus oryzae* is the essential microorganism in the preparation of the Indonesian fermented soybean food, tempeh. Development of bacteria in the fermenting soybeans was undesirable, contributing off-odors and flavors. Essential for satisfactory growth of the mold on the beans were: (1) removal of the skins from the beans, which, by mechanical means, has proved to be difficult; (2) adjustment of the pH of the beans to within a range of 4.0 to 5.0 which inhibits growth of bacteria, but does not interfere with mold growth; (3) sufficient access of oxygen for mold growth through the bean mass; (4) sufficiently high humidity to prevent any drying of the surface of the beans; and (5) an initial temperature of 37° C in the beans, controlled so as not to exceed 45° C during the fermentation.

As the fermentation progressed, soluble solids rose from 13 to 27.5%. Total nitrogen remained constant, at approximately 7.5%, while soluble nitrogen rose from 0.5 to nearly 2.0%. The pH of the fermenting beans gradually rose from 5.0 to 7.6. Reducing substances showed an over-all decline during the fermentation. The fiber content increased from an average of 3.7% in the skinned beans to 5.85% in the tempeh due to development of mold mycelia.

Sectioned beans from tempeh showed only slight penetration of the mycelia into the underlying tissues of the bean. This suggests that the bean digestion is mainly enzymatic. The fermented beans are softened, similar to thoroughly cooked soybeans. When beaten in a Waring blender with water, the individual cells resisted fracture and remained intact. In contrast, the raw soaked beans, given similar treatment in the blender for even 30 sec, retained relatively few intact cells.

Tempeh was considered to be at its optimum, organoleptically, when the pH reached 6.5 and the soluble solids content was approximately 21%. Tempeh was preserved by drying. Lyophilization had less effect upon the soluble components in the tempeh than air drying.

The yield of tempeh by the procedures described was approximately 72.5 g (dry substance) for each 100 grams starting soybeans (dry weight).

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EFFECT OF CURE ON PORK WITH WATERY STRUCTURE. I. BINDING OF SALT AND WATER TO THE MEAT

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(Manuscript received October 13, 1959)

In an earlier report *(13)* properties of pork in relation to the fall in pH immediately post mortem were considered. A rapid fall in pH imparted a watery structure to the meat through partial denaturation of the proteins. The implications of this structural change on the curing process and the ability of the meat to absorb pickle will be considered. Denaturation manifests itself in a decreased solubility of the proteins, and one may therefore expect a decreased water binding capacity since solubility and the water binding capacity of meat proteins are intimately associated *(8)*. However, the effect of denaturation of proteins on their solubility is quite variable, depending on the actual pH of the proteins, and may not be observed unless the pH is close to the isoelectric point.

The process of curing, however, involves more than the meat proteins. Interaction between meat proteins and cell membranes can also be expected. The uptake of pickle by comminuted meat as well as by whole meat cuts has therefore been studied. As a criterion for the state of the cell membranes, an estimation of the concentration of the intracellular substance desoxypentose nucleic acid in the free meat juice was used.

ANALYTICAL PROCEDURES

Determination of pH. This was done as described earlier *(13)*. The pH was measured ¾ hr after sticking the animal (pH_1) and when the measurement of water binding capacity took place or the meat was cured (pH_2).

Determination of water holding capacity. This was accomplished as described earlier *(13)*. The water holding capacity is expressed by the LW value which designates the amount of fluid in 1 mg which can be pressed out of 1 g of meat under standardized conditions.

Determination of water binding capacity. The meat sample was ground twice through a household grinder fitted with a disk with 4 mm holes; 20 g of the ground meat were mixed with 20 cc of 5% NaCl solution in a 60 cc centrifuge tube, and the mixture was stored at 4° C for 16 hr. The mixture was then centrifuged at 1500 g for 20 min and the volume of the centrifugate was subsequently measured.

Determination of NaCl. This was made by precipitation with silver nitrate *(2)*.

Determination of desoxypentose nucleic acid (DNA). The fluid in which the DNA was determined was collected from samples of the same size as those used for the salt diffusion experiments. Samples were placed between plexiglass plates and gently pressed. Several samples were often used as the pressing continued until 5 ml of press fluid was obtained. The method of separation of the DNA was that described by Love *(10)*. The DNA was determined through the content of phosphorus. The phosphorus was determined according to Allen *(1)*.

Determination of potassium. This was done according to the method described by Eden *(7)*.

Determination of sodium. Sodium was determined as described by Ludvigsen *(11)*.

EXPERIMENTAL MATERIAL

The meat for these studies was taken from *longissimus dorsi* and *adductor* of carcasses from Landrace pigs reared at the progeny testing station "Sjaelland" under

identical conditions. Pigs were slaughtered at Roskilde bacon factory after electrical stunning. The meat structure was studied 24 hr after slaughter. The distinction between normal and watery meat structure was described earlier *(13)*.

RESULTS

Water binding capacity of meat proteins. Results of the water binding determinations of 24 samples of *longissimus dorsi* after thorough grinding are given in Table 1. Each sample represents an individual carcass. Sample was taken from the muscle at a position corresponding to the last rib. Meat samples had pH_2 values between 5.2 and 5.4 when the determinations were made, which means close to the isoelectrical point of the meat proteins. The samples were divided into two groups in accordance to the pH_1 values. As shown earlier *(13)*, pH_1 values below 6.2 are associated with the watery structure of the meat. The watery structure is illustrated by LW values considerably

TABLE 1

Effect of structure on water binding capacity of ground meat

	Sample No	LW	pH_1	Weight of bound pickle in % of meat weight	mg N diffused into solution from 20 g meat	% NaCl in meat
	1	515	.....	1	147	2.38
	2	515	.....	5	146	2.36
	3	512	5.50	3	137	2.38
	4	511	5.56	14	136	.....
	5	507	.....	3	138	2.38
Meat with	6	503	5.52	6	141	2.41
watery	7	498	5.51	5	144	.....
structure	8	492	5.44	0	142	2.41
	9	489	5.79	28	128	.....
	10	487	5.41	5	136	.....
	11	467	5.60	10	138	.....
	12	457	5.66	5	143	.....
	Average	496		7.1	139.7	2.39
	13	484	6.21	32	114	.....
	14	449	6.57	47	103	.....
	15	448	6.33	26	139	.....
	16	446	.....	48	104	2.48
	17	441	6.32	45	106	.....
Meat with	18	440	.....	33	136	2.45
normal	19	435	.....	59	82	2.47
structure	20	435	6.44	26	133	2.49
	21	432	6.38	79	48	2.53
	22	431	6.23	34	121	.....
	23	425	6.61	28	139	.....
	24	.....	6.27	28	126	2.47
	Average	442		40.4	112.6	2.48

above 460. The water binding capacity was observed to be much lower in the meat with the watery structure than it was in the group which we shall call normal. The watery meat could, on the average, only absorb 7.1% of the salt solution compared to 40.4% for the meat of normal structure. Results of the determinations of the salt content show that the concentration was only slightly lower in the meat with the watery structure. Thus, the difference in the water binding capacity can hardly be due to the specific salt effect on the water binding capacity of meat *(9)*. The determination of nitrogen shows that more nitrogenous compounds have diffused out of the watery meat than out of the normal tissue. The difference, however, is not so great as the difference in the water binding capacity.

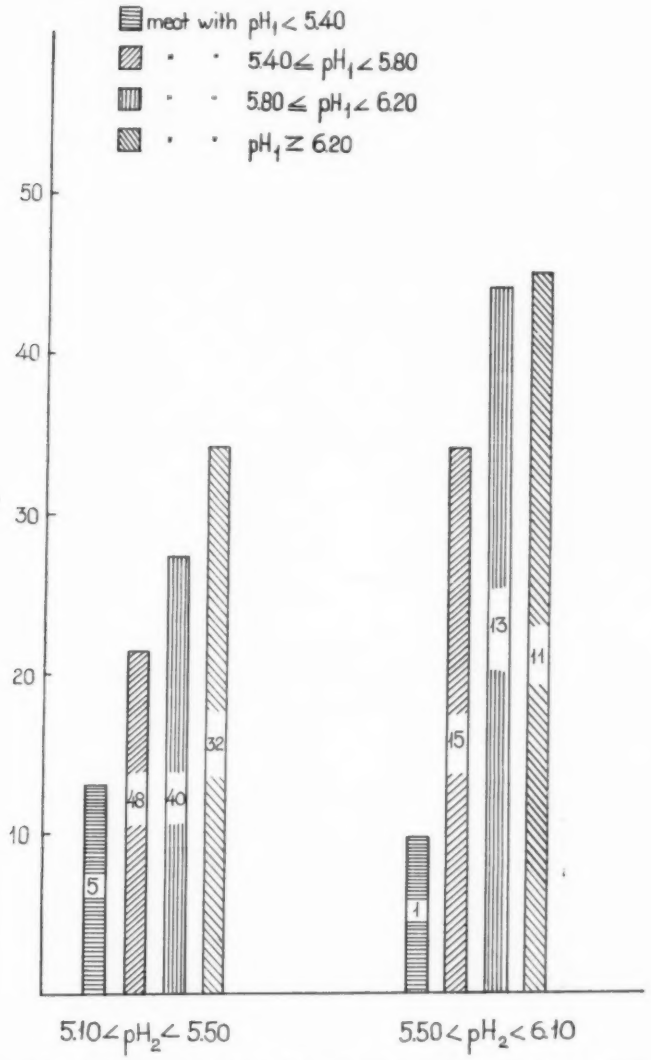

Figure 1. Absorption of pickle in relation to pH_1 and pH_2 of the meat samples.
The numbers on the columns denote numbers of observations.

These results are in accord with the concept that watery meat contains proteins in a more or less denatured stage. But, as mentioned earlier, the effect of denaturation may not be of importance when the pH_2 of the sample does not correspond to the isoelectric point. In order to study this question, the water binding capacity was determined on 165 samples of *longissimus dorsi* and *adductor* muscles with pH values scattered from 5.1 to 6.1. The results are shown in Figure 1. Samples were divided into 4 groups

with regard to the pH_1 values and 2 groups with regard to the pH_2 values. The columns give the average water binding capacity for the groups. It is of interest that the lower the pH_1 values, the lower the water binding capacity. When samples with pH_2 around the isoelectric point are considered there is a uniform decrease in the water binding capacity with lower pH_1 values. For samples with the higher pH_2 values the differences between the groups are diminished, which shows that the effect of the rate of pH decline post mortem on the water binding capacity of the meat levels out when pH_2 remains above 5.5. All these samples have, on the average, higher water binding capacities—reflecting the general improving effect of higher pH on the water binding capacity of meat proteins. The only exception is the single observation in the group with $pH_1 < 5.4$, where pH_2 was found to be 5.5.

Water binding capacity of meat cuts. The water binding capacity of whole meat as opposed to ground meat may not be a direct function of the water binding capacity of the meat proteins. Callow's work *(3, 4, 5, 6)* on the importance of meat structure in relation to the cure hinted that there were no direct relationships between the water binding capacity of the meat proteins and uptake of pickle in the cured pork. We have studied the water binding capacity of meat cuts with pH_2 in the region of 5.2 to 5.5. In this pH range the greatest variation was in the water binding capacities of the proteins. As the range is rather narrow, the results are quite undisturbed by the well-known pH effect of salt absorption, etc., during the cure *(12)*. For this study model experiments were designed, using sections of *longissimus dorsi* cut out of the muscle at the last rib. Each section was 30 g in weight. Before beginning the cure, the intactness of the cell membranes was studied.

The aim of the experiment was to study the effect on the cell membranes. When one observes the rather excessive exudate which oozes out of watery pork one might think that the cell membranes are more or less ruptured. In order to find out the extent to which this is true, we estimated the concentration of desoxypentose nucleic acid (DNA) in the fluid which diffuses out of the meat under gentle pressing. DNA is a fairly high molecular compound characteristic of the intracellular fluid.

TABLE 2

Estimation of DNAP content of fluid expressed from meat with watery and normal structure

	Sample No	pH_1	mcg DNAP in 5 ml of fluid
Meat with watery structure	1	5.30	12.4
	2	5.30	12.0
	3	5.40	20.5
	4	5.49	13.4
	5	5.55	12.4
	6	5.59	17.8
	7	6.15	13.6
	Average		14.6
Meat with normal structure	8	6.47	12.0
	9	6.69	10.4
	10	6.69	9.6
	11	6.74	18.3
	12	6.74	9.6
	Average		12.0

Results are presented in Table 2. Each sample represents an individual pig. DNA content is given as mcg of the DNA phosphorus (DNAP) in the fluid. There is considerable variation between the individual samples, due, perhaps, to damage of the cell membranes during sample preparation. As a rule, the concentration of DNAP is a little higher in the press fluid from meat with watery structure, indicating the membranes are more permeable to cell constituents in watery meat than is true in normal meat. However, the difference in the concentration of DNAP between the meat

TABLE 3

Effect of grinding on DNAP content in expressed fluid from meat with normal structure

	mcg DNAP in 5 ml of fluid
30 g sample	11.3
After grinding once	17.4
After grinding twice	58.2

with normal and that of watery appearance was not considerable when compared to the concentration obtained in the fluid after grinding the meat twice (Table 3). When the meat was ground twice, the cell membranes were considered ruptured since further grinding or homogenization did not provide higher DNAP values. Therefore, we drew the conclusion that the cell membranes in watery pork were somewhat more permeable to DNA than the membranes in normal meat. But by and large they were still intact. With regard to the samples the cell membranes were considered so intact that the effect of membranes may be demonstrated when the results of salt and water uptake of the samples were compared to those obtained with the ground meat.

For the curing experiment, each of the samples mentioned above was placed in a Petri dish with a quantity of pickle of the same weight as the meat sample. The pickle was a sodium chloride solution of a strength between 5 and 10%. The dish with meat and pickle was placed at 4° C for 16 hr; the solution was then drawn off the meat and measured. The meat and the solution were analyzed for salt and nitrogen content.

The results are given in Table 4. Each sample represents an individual pig. The meat with watery structure bound less water, it was observed, than the normal meat, and more nitrogeneous compounds diffused out of the meat, as was noted in the experiments with ground meat. However, the salt content in the meat was consistently higher when the structure was watery. This observation suggests an effect which might be due to the cell membranes since the salt content was consistently lower in the samples of watery meat when studied after grinding. The effect of the increasing strength of the salt solution was less water uptake by the meat, as might be expected, and a progressive increase in the salt uptake of the meat. The effect of the increasing ionic strength of the pickle is also illustrated through the increased flow of nitrogen compounds out of the meat. The normal and the watery meat appear to react similarly to the increasing concentration of the pickle and hence their osmotic properties appeared rather equal.

In the curing process the extracellular as well as the intracellular fluids may take part. In order to study the extent to which there may be differences between the two kinds of pork in this respect, we measured the diffusion of potassium and sodium ions between the meat samples and the salt solution. In fresh meat, potassium is mainly located within the cells and the sodium between the cells. The relative movements of these ions may provide an illustration of the movement of the extracellular and intracellular fluids. Results are given in Table 5 for a curing experiment similar to that given in Table 4. Samples were immersed in the same weight of 5% sodium chloride solution. Sodium chloride of analytical grade (Merck) was used. For the fresh meat we found the same concentration of potassium in both categories of meat and for sodium slightly less in the watery meat. After cure, however, the potassium content was considerably lower in the watery meat, indicating that the potassium had diffused out of this meat to a greater extent than from the normal meat. As far as the sodium ions are concerned, results reflected what was implied in the determination of the total salt content; the concentration was higher in the meat with watery structure. This seems to indicate that the higher concentration of salt in the watery meat is due to a more thorough diffusion of the salt solution into the cells.

In general, when watery pork is cured, the meat juices diffuse *out* and the salt solution diffuses *into* the meat to a greater extent than when normal meat is cured.

When whole muscles are cured, the higher absorption of salt may be associated with a higher gain in weight during cure for meat with watery structure. We have conducted an experiment with tenderloins *(psoas)* which were dissected out of the carcasses as carefully as possible and cured 24 hr after slaughter. The cure took

TABLE 4

Effect of structure on the absorption of salt and water by meat samples

	Sample No	pH_1	LW	% weight gain during cure	mg N diffused into the salt solution	Total chloride in the meat as % NaCl	% NaCl in the solution	Initial strength of salt solution in %
Meat with watery structure	1	5.50	512	11	70	1.67	4.26	
	2	5.44	492	6	71	1.54	4.32	5.00
	3	5.52	503	7	58	1.58	4.54	
	Ave		502	8	67	1.60	4.37	
	4	5.50	507	0	113	2.04	5.46	
	5	5.56	501	0	118	2.13	5.35	6.00
	6	5.43	491	3	109	2.15	5.37	
	Ave		500	1	113	2.11	5.39	
	7	5.50	507	2	110	4.00	7.68	
	8	5.56	501	0	116	4.27	7.59	10.00
	9	5.43	491	1	106	4.09	7.63	
	Ave		500	1	111	4.12	7.63	
	Combined Average			3	97	2.61	5.80	
Meat with normal structure	10	6.38	432	8	55	1.51	4.59	
	11	6.44	435	9	61	1.40	4.29	5.00
	12	6.27	.....	14	42	1.51	4.15	
	Ave		434	10	53	1.47	4.34	
	13	6.88	442	7	92	1.92	5.53	
	14	6.66	432	6	100	2.01	5.53	6.00
	15	6.61	431	6	91	1.96	5.53	
	Ave		435	6	94	1.96	5.53	
	16	6.88	442	3	91	4.12	7.59	
	17	6.66	432	5	104	4.09	7.70	10.00
	18	6.61	431	5	106	3.90	7.84	
	Ave		435	4	100	4.04	7.71	
	Combined Average			7	82	2.49	5.86	

place in beakers with an amount of 20% sodium chloride solution corresponding to the weight of the muscle. The beakers were placed at 4° C for 16 hr. The results are given in Table 6. Each sample represents an individual pig. A rather considerable difference in the average gain in weight is seen, although considerable variation exists within the two groups. Similar observations on the effect of watery structure on the salt uptake of meat have been made in commercial cures of canned hams and Wiltshire bacon.

It may be of interest to know how firmly the water is bound in the cured meat in relation to the structure. We have tried to study this question by determining how much water can be pressed out of the samples before and after cure. The determinations were made in the same way as the determinations of the water holding capacity of fresh meat. Results are expressed in mg of water pressed out of 1 g of meat. Figure 2 shows the results for two typical examples of normal and watery meat. The samples were 200 g sections of *longissimus dorsi* cured with the same weight of 5% sodium chloride solution in the beakers. The beakers were placed at 4° C for 44 hr. Then the salt solution was drawn off and the samples "matured" in a Petri dish for 5 days. One may observe that the amount of water which can be pressed out of the meat goes down during the time of maturation due to the well-known effect of salt on the water binding

TABLE 5

Diffusion of sodium and potassium during cure

	Sample No	pH_1	pH_2	I.W	% gain in weight in cure	mg N diffused into salt solution	Total salt in solution after cure determined as chloride mg/g	Total salt in meat after cure determined as chloride mg/g	Sodium in fresh meat mg/g	Sodium in cured meat mg/g	Potassium in fresh meat mg/g	Potassium in cured meat mg/g
Meat with watery structure	1	5.36	5.31	519	3	125	28.7	22.0	0.75	8.80	3.54	1.34
	2	5.48	5.44	473	5	106	26.3	24.6	0.79	10.53	3.48	1.37
	3	5.70	5.24	513	1	127	23.5	23.5	0.79	9.54	3.25	1.17
Average				502	3	119	26.2	23.4	0.77	9.62	3.42	1.29
Meat with normal structure	4	6.31	5.46	436	8	107	29.2	20.8	0.87	8.71	3.45	1.55
	5	6.57	5.44	430	14	130	27.6	24.1	0.90	7.94	3.38	2.11
	6	6.65	5.50	377	11	135	26.3	22.2	0.69	9.99	3.43	1.31
Average				414	11	124	27.7	22.4	0.82	8.88	3.42	1.66

TABLE 6

Weight gain during cure of m. psoas

	Muscle No	pH_1	pH_2	% gain in weight during cure
Meat with watery structure	1	5.61	5.45	11.1
	2	5.70	5.52	10.0
	3	5.83	5.44	5.1
	4	5.87	5.56	3.6
	5	6.09	5.40	5.2
				7.0
Meat with normal structure	6	6.20	5.40	2.9
	7	6.28	5.53	4.7
	8	6.30	5.58	1.6
	9	6.33	5.50	2.3
				2.9

capacity of meat proteins. In comparing the two structures, the most water could be pressed out of the meat with the watery structure. Along with the determinations of the water holding capacity the nitrogen content and pH of the meat were determined. The pH values were uniform for both samples. Results of the nitrogen determinations are shown on the figure. In the fresh meat the content was slightly higher in the meat with watery structure indicating a slightly lower water content. The difference was diminished when the samples were cured, and during the maturation period the nitrogen content in the watery meat was slightly lower—reflecting the tendency to increased interchange of pickle and nitrogenous compounds in the meat with watery structure. The results appear to indicate that water absorbed during cure is more loosely bound in the meat with watery structure than in meat of normal structure.

DISCUSSION

The low water binding capacity of the meat proteins in the watery pork occurred around the isoelectric point—that is, in the pH range of about 5.1 and 5.5. In the higher pH interval, where the watery structure in pork may occur, effect on the water binding capacity of the meat is far less noticeable. This observation may prove of interest in the manufacture of emulsion type sausages, where good quality is highly dependent on good water binding capacity. When pork is used as raw material and meat with watery structure likely to be the result, it seems mandatory to avoid meat with a pH between 5.1 and 5.5. Use of polyphosphates in the emulsion may to a certain extent improve the water binding capacity of the watery meat but to a much lower degree than it improves the water binding capacity of normal meat *(14)*.

When meat cuts of watery structure are cured, meat juices and salt solution are exchanged in much greater degree than is the case with normal meats. This exchange leads to an increased salt content in the meat. When whole muscles or large cuts are cured, there is an increase in weight above normal. The absorbed salt solution is not, however, firmly bound in the meat and the water holding capacity of cured meat of watery structure is thus lower than with cured meat of normal structure. The difference in the salt absorption between normal and watery meat may be regarded as a "sponge" effect.

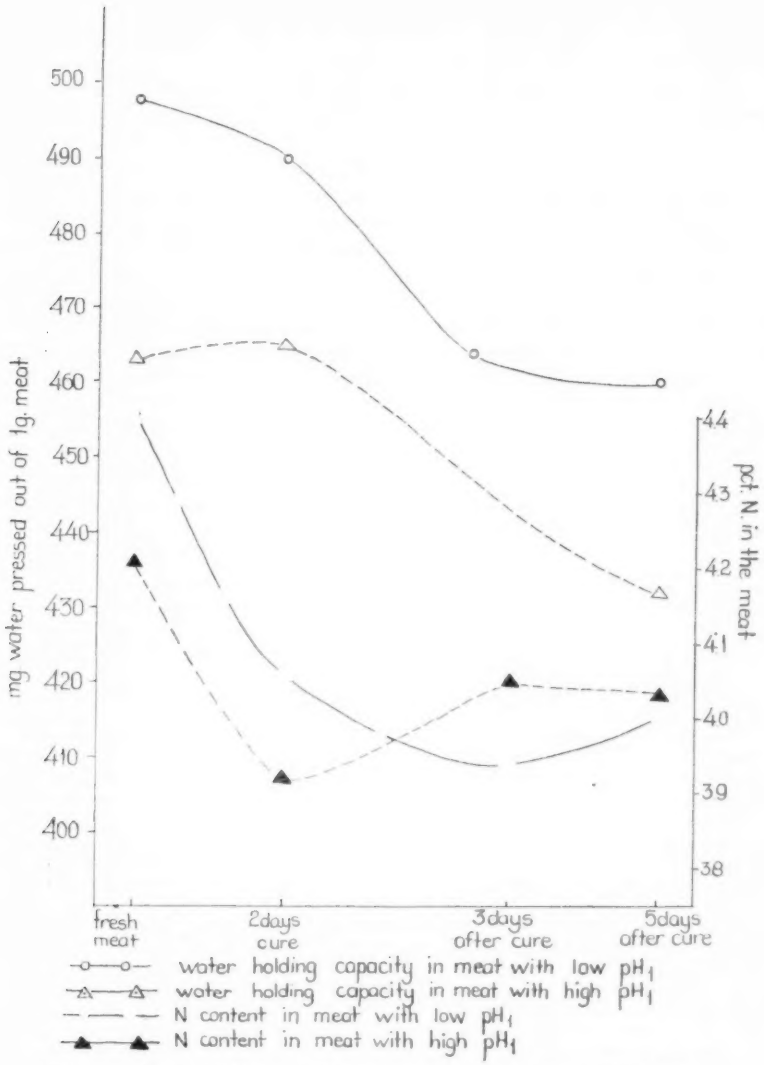

Figure 2. Water binding capacity of normal and watery meat during cure.

The watery structure considered in this paper is similar, although not identical with the open structure that Callow obtained by cooling *psoas* muscles slowly to 0° C versus the closed structure effected by rapid cooling *(5)*. The salt absorption in Callow's experiment was the highest for the meat with open structure, but loss of soluble matter from the muscular tissue was less. These results were obtained when the meat was covered with salt

or immersed in a salt solution. When the meat was covered with dextrose instead of salt, more soluble matter was lost from the meat with open structure than from that with closed structure.

The watery structure thus appears to be more open than the open structure so widely investigated in the 1930's.

SUMMARY

The capacity of pork with watery structure to bind salt and pickle has been studied. When ground, the watery meat had a low water binding capacity—especially when the pH was close to the isoelectric point of the meat proteins.

When whole cuts were covered, the watery meat absorbed more pickle than normal meat. The diffusion of salt into the meat and nitrogeneous compounds out of the meat was higher for the watery meat than for that with normal structure. Studies on the flow of K, Na and DNA indicated a more thorough penetration of the pickle into the meat cells of the watery meat compared to the normal meat.

Acknowledgment

The author is indebted to Mrs. I. Rosenbom for her very competent and careful help in the analytical work.

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EFFECT OF CURE ON PORK WITH WATERY STRUCTURE. II. EFFECT ON QUALITY OF CANNED HAMS

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Differences between normal and watery pork in relation to the curing process were discussed in Part I. It was found that watery meat took up more salt and lost more meat juice during the cure than normal meat. We shall now try to determine the extent to which these differences affect the quality of cured meat products after canning. For this purpose we have made some observations on canned hams.

The quality of canned hams may vary considerably with variations in the raw material. In addition, the principal muscles processed for use as canned hams may differ in water holding capacity as between pigs. The variations are similar to those in the loin muscle. The results of determinations of water holding capacity and pH_1 for *m. adductor* give useful indications of the quality of the ham musculature in general.

The impact of the watery structure on the quality of the final products may depend on the extent to which the meat is heated during processing. If the meat is heated to make the product sterile in the can, effects of the structural differences between the normal and watery pork may be eliminated. On the other hand when the product is only pasteurized the effects of the differences in the meat structure may be of importance for the quality of the product.

EXPERIMENTAL MATERIAL

The experimental material consisted of 79 hams from Danish Landrace pigs (live weight, 90–110 kg). Pigs were slaughtered at Roskilde bacon factory after electrical stunning. The pH was measured ¾ hr after slaughter (pH_1) in *m. adductor;* 24 hr after slaughter the meat structure was examined and the hams cured. The hams were stitch pumped with a 17° B pickle containing salt, $NaNO_3$, $NaNO_2$ and sodiumtripolyphosphate. The hams were tank cured for 4 days and matured for 4 days; 50 of the hams were then processed to whole pasteurized hams. They were packed in B cans of a size corresponding to the weight of the individual ham and evacuated. The hams were given a heat treatment until the center had a temperature of 64° C. Of the remaining 29 hams samples with a weight of 2 lb were cut out. The samples were packed in 2 lb pearshaped cans and evacuated. The cans were given a heat treatment until the center had a temperature of 102° C.

Analytical procedures. *Organoleptic evaluation.* Each ham was subjected to a taste panel of 5 to 7 experienced people. Scores were given for colour, taste and texture of the meat. For the scoring a scale of 10 was used: 10 denoting *excellent,* 8 *good,* 6 *minor quality deficiency,* 4 *quality deficiency,* 2 *strong quality deficiency* and 0 *extremely bad quality.*

Determination of salt was made by precipitation with silver nitrate *(1).*

Determination of nitrosomyoglobin was made as described earlier *(2).*

RESULTS AND DISCUSSION

Table 1 gives the results of the quality evaluation and the chemical analysis of the hams that underwent the light heat treatment. For the sake of brevity only the average results are given for the two groups (meat

TABLE 1

The quality of pasteurized hams in relation to meat structure

	Number of observations	Scores for				Nitroso-myoglobin mg /10 g meat	% jelly in can
		Colour	Taste	Texture	Salt		
Meat with watery structure	27	6.68 (0.81)	7.40 (0.64)	7.22 (0.76)	3.32	36	11.8
Meat with normal structure	23	7.17 (0.69)	7.54 (0.57)	7.36 (0.60)	3.17	38	10.8

Standard deviations are given in parentheses.

with watery structure and meat with normal structure). The distinction between the two groups was made through measurement of pH_1 and water holding capacity of *m. adductor*, as explained in Part I.

Colour, taste and texture were inferior, it was noted, in the hams from meat with watery structure. Differences in the colour scores were most pronounced. The colour was generally paler in the hams from watery meat. The difference is statistically significant at $P = 0.05$. The paleness must be considered due to opaqueness of the meat fibers, resulting from the more advanced degree of coagulation. Results of nitrosomyoglobin determinations show that the pale colour is not due to a diminished pigment concentration since that appears to be equal for both groups. The difference in taste and texture does not reach statistical significance. It may serve, however, as an illustration of the effect of the higher exchange of meat juices and pickle on product quality. The salt content of the watery hams is, as expected, slightly higher than the salt content of the normal hams.

In Table 2, corresponding results for the sterilized hams are given. The stronger heat treatment has resulted in a considerably higher percentage of jelly in the can due to a more advanced stage of coagulation of the meat proteins. One may note that there is no difference between the two groups as far as colour, taste and texture are concerned. Obviously, the heat treatment has been so considerable that the degree of coagulation of the proteins

TABLE 2

The quality of sterilized hams in relation to meat structure

	Number of observations	Scores for				Nitroso-myoglobin mg /10 g meat	% jelly in can
		Colour	Taste	Texture	Salt		
Meat with watery structure	18	7.06 (0.69)	7.16 (0.46)	6.93 (0.66)	2.80	39	29.6
Meat with normal structure	11	7.11 (0.48)	7.11 (0.29)	6.89 (0.43)	2.99	36	29.9

Standard deviations are given in parentheses.

has become equal. So much meat juice has cooked out that the slight effect on taste and texture of the differences in the cure between the two categories of meat noticed in the pasteurized hams has disappeared.

SUMMARY

In a study of the effect of cure on the quality of canned hams the effect of the differences in salt absorption and diffusion of meat juices during cure of normal and watery meat was examined. The canned hams were studied in perishable as well as sterile condition. For the perishable hams an adverse effect of watery meat on quality was observed, mainly as regards colour. When hams were cooked to commercial sterility, there was no difference between those processed from normal and those from watery meat.

Acknowledgment

The author is indebted to staff members at the Danish Meat Products Laboratory for assistance in the quality evaluation of the hams. Thanks are due to Mr. Ove Madsen for the salt determination.

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A NEW MODEL IN CONSUMER TESTING

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When a pair of products, A and B, are consumer tested for preference, it is a well-recognized phenomenon that many of the respondents indicate a preference (even though they really have none, and should be indicating "no difference"), simply because they are in a test situation and therefore try a little too hard. At times like these they are influenced by external factors such as order of tasting effect, or bias in the coding of the samples, into indicating a pseudo-preference for one or other of the samples. The analysis below enables the estimation of preference proportions for the products, A and B, corrected for such pseudo-preferences.[a]

TEST PROCEDURE AND QUESTIONNAIRE

A sample of product A and a sample of B are presented to each of 4N consumers for preference evaluation. It is assumed that these 4N consumers form a representative sample of the consumer population at whom products A and B are eventually aimed and that samples of products A and B used in the test are representative and homogeneous. In getting 2N of these judgments the sample of A is presented first; in the other 2N judgments the sample B is tasted first. This balances out the order bias in the overall results. Similarly, within each set of 2N, the sample of A bears a given code-letter, or code number, or code symbol, N times, B bears the same letter or number or symbol the other N times. It is assumed that the code numbers, letters, or symbols are judiciously chosen so as not to be particularly suggestive.

Thus, summarizing, the 4N judgments are obtained as follows:

(Suppose code numbers 563 and 567 are used).

N judgments where the sample of A bears the "lower" code designation (A = 563, B = 567) and is tasted first,

N judgments where the sample of A bears the "higher" code designation (A = 567, B = 563) and is tasted first,

N judgments where the sample of A bears the "lower" code designation (A = 563, B = 567) and is tasted last,

N judgments where the sample of A bears the "higher" code designation (A = 567, B = 563) and is tasted last.

(There is a belief in some circles that the suggestive sequentiality of code numbers is much reduced if 3 digits or more are used. While not particularly believing this, I have used such code numbers for illustration in this paper. Suggestion-free numbers, letters, symbols, or colors yet remain to be discovered.)

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[a] The difference between the present treatment and that in an earlier publication by the author (see *1*) consists of getting all the necessary data at a single "visit" to members of the consumer panel. It is assumed that at the given instant of the test a consumer can or cannot discriminate between the pair of samples, does or does not have a preference—with no shades of grey in between. There is no attempt to characterize consumers by how they might behave during subsequent tests, as was done in the earlier paper. The problem of estimating the complete distribution of abilities to discriminate together with the distribution of degrees of preference in respect of a pair of products still remains to be solved.

The questions asked (orally or in writing) are as follows, apart from introductory and explanatory remarks:

Question 1: Do you think these samples are alike or different?

Alike ..... Different .....

(Stop here if the answer "Alike" is given, and record the vote as Type O.)

Question 2: If you think the samples different, do you have a preference or do you like (or dislike) the samples equally?

Have a preference ..... Like (dislike) equally .....

(Stop here if the answer "Like (dislike) equally" is given, and record the vote as Type E.)

Question 3: If you have a preference, which of the two samples do you prefer?

563 ..... 567 .....

(Record the vote as Type A or Type B, after decoding for the particular judgment.)

At the end of Question 3 one may ask an additional question concerning degree of preference; i.e., one may quantify the degree of preference for purpose of a Scheffé analysis (see 2) with or without transforming the actual scale used.

The model assumed. (1) It is assumed that with regard to the pair of products A and B, there is a proportion p of consumers who can discriminate.

(2) It is assumed that of this proportion p of discriminators π_a prefer A, π_b prefer B, π_e have equal preference.

(3) It is further assumed that among the proportion (1−p) of non-discriminators a proportion q are susceptible to influence in the test situation by order of presentation and coding of the samples, thereby being led to behave like pseudo discriminators.

(4) The remaining proportion (1−p−q) will always behave like non-discriminators (i.e., admit it).

Summarizing the model in Table 1 below:

TABLE 1

The model

p discriminators			(1 − p) non-discriminators	
π_a prefer A	π_b prefer B	π_e have equal preference	q pseudo discriminators	(1−p−q) behave as non-discriminators

Expectation of various vote-types. In Table 2 below are entered the *relative* frequencies of the various types of judgments, as *observed* during a given test with 4N judgments, say. Marginal observed frequencies are also designated.

Before giving formulae for the expectations of (1) to (16) in Table 2 under the proposed model, two quantities r and s will be defined as follows: r is the bias in favor of the first tasted sample, estimable from the data, expressed as a ratio ($r > 1$ means an actual bias in favor of the first-tested sample, $r < 1$ a bias in favor of the last). s is the bias in favor of the "lower" code, estimable from the data, expressed as a ratio

TABLE 2

Observed votes

Type of vote	A first A low	A first A high	A last A low	A last A high	Total
O	(1)	(5)	(9)	(13)	(17)
A	(2)	(6)	(10)	(14)	(18)
B	(3)	(7)	(11)	(15)	(19)
E	(4)	(8)	(12)	(16)	(20)
Total relative frequency:	1	1	1	1	4

($s > 1$ means an actual bias in favor of the "lower" code, $s < 1$ a bias in favor of the "higher").

It is assumed that order and code biases are multiplicative in the ratio sense defined. Expectations are given in Table 3.

TABLE 3

Expectations of observations (Table 2)

$$E(1) = E(5) = E(9) = E(13) = 1 - p - q$$
$$E(4) = E(8) = E(12) = E(16) = p\pi_e$$
$$E(2) = p\pi_a + q\frac{rs}{2}$$
$$E(3) = p\pi_b + q\left(1 - \frac{rs}{2}\right)$$
$$E(6) = p\pi_a + q\frac{r}{2s}$$
$$E(7) = p\pi_b + q\left(1 - \frac{r}{2s}\right)$$
$$E(10) = p\pi_a + q\frac{s}{2r}$$
$$E(11) = p\pi_b + q\left(1 - \frac{s}{2r}\right)$$
$$E(14) = p\pi_a + q\frac{1}{2rs}$$
$$E(15) = p\pi_b + q\left(1 - \frac{1}{2rs}\right)$$

Estimation of the parameters. The maximum likelihood estimates of the parameters will next be obtained. For simplicity, the ML estimates of the parameters p, q, r, s, π_a, π_b, and π_e will be denoted by the same symbols in the formulae below (instead of the usual p, q, ... etc). It is also clear that π_e is not independent, as $\pi_e = 1 - \pi_a - \pi_b$, and so there are only six independent parameters.

The ML function is given by:

$$\begin{aligned} L = {} & [(1) - (1-p-q)]^2 + [(5) - (1-p-q)]^2 + [(9) - (1-p-q)]^2 \\ & + [(13) - (1-p-q)]^2 + \left[(2) - \pi_a p - \frac{rs}{2}q\right]^2 + \left[(6) - \pi_a p - \frac{r}{2s}q\right]^2 \\ & + \left[(10) - \pi_a p - \frac{s}{2r}q\right]^2 + \left[(14) - \pi_a p - \frac{1}{2rs}q\right]^2 \\ & + \left[(3) - \pi_b p - \left(1 - \frac{rs}{2}\right)q\right]^2 + \left[(7) - \pi_b p - \left(1 - \frac{r}{2s}\right)q\right]^2 \\ & + \left[(11) - \pi_b p - \left(1 - \frac{s}{2r}\right)q\right]^2 + \left[(15) - \pi_b p - \left(1 - \frac{1}{2rs}\right)q\right]^2 \end{aligned}$$

There is no need for the dependent terms involving (4), (8), (12), and (16). The six ML equations are obtained by the usual partial differentiation of L with respect to the 6 parameters. For simplicity, write:

$$x = rs \qquad y = \frac{r}{s} \qquad t = \frac{(r^2+1)(s^2+1)}{2rs}$$

Then, the six equations are as follows:

Equation I. (Partial differentiation with respect to π_a):

$$\pi_a = \frac{(18) - qt}{4p}$$

Equation II. (Partial differentiation with respect to π_b):

$$\pi_b = \frac{(19) - q(4 - t)}{4p}$$

Equation III. (Partial differentiation with respect to p):

$$p = 1 - \tfrac{1}{4}(17) - q$$

Equation IV. (Partial differentiation with respect to q):

$$q = \frac{\frac{t}{2}[(19)-(18)] + rs[(2)-(3)] + \frac{r}{s}[(6)-(7)] + \frac{s}{r}[(10)-(11)] + \frac{1}{rs}[(14)-(15)]}{\frac{(r^4+1)(s^4+1)}{r^2s^2} - t^2}$$

From the above four equations I to IV one may express p, q, π_a, and π_b in terms of r and s. Hence, if two more equations involving r and s are obtained, the problem is theoretically solved.

Obtaining $\frac{1}{rq}\frac{\delta L}{\delta s}$ and $\frac{1}{sq}\frac{\delta L}{\delta r}$, then adding (Equation V) and subtracting (Equation VI) the resulting expressions:

Equation V.

$$qx^4 + [(3) - (2) + (\pi_a - \pi_b)p - q]x^3 + [(14) - (15) - (\pi_a - \pi_b)p + q]x - q = 0$$

Equation VI.

$$qy^4 + [(7) - (6) + (\pi_a - \pi_b)p - q]y^3 + [(10) - (11) - (\pi_a - \pi_b)p + q]y - q = 0$$

These expressions V and VI appear rather formidable, but the relevant solutions of r and s can be guessed from the model, and after considerable algebra can be shown to satisfy V and VI.

They are:

$$r^2 = \frac{(2) + (7) - (3) - (6)}{(10) + (15) - (11) - (14)}$$

$$s^2 = \frac{(2) + (11) - (3) - (10)}{(6) + (15) - (7) - (14)}$$

These last two expressions (and the derived t) are then substituted in IV to give q, this in turn gives p from III, whence π_a and π_b follow from I and II.

Estimated variance-covariance matrix of the estimates. The inverse of the matrix formed by the second derivatives of the function L is required. These second derivatives are given in Table 4 below. In practical cases estimates would be substituted for the parameters in the various derivatives.

DEPARTURES FROM THE MODEL AND OTHER DIFFICULTIES

Before proceeding to illustrate the model with a numerical example, it may be advisable to discuss certain features of the model and the estimation procedure.

1. Multiplicativity of order and code bias. This is a reasonable assumption in any case, and here a necessary one for a solution. Additive r and s assumed in the model will lead to identifiability difficulties.

2. Constant q for the 4 groups of N judgments. One can make the proportion of pseudo-discriminators q a function of the situation that confronts consumers; i.e., one may use q_1, q_2, q_3, and q_4 instead of a single q, for groups of judgments A first A low, A first A high, A last A low, A last A high, respectively. If the observations (1), (5), (9) and (13) are significantly different, this is an indication that the model with a single q is not valid. The estimation procedure follows exactly as in the case of a single q. Normally, one would not wish to use 9 parameters (4 q's) if 6 parameters (one q) fit the data.

TABLE 4
Second derivatives

$$\frac{\delta^2 L}{\delta p^2} = S\,(1 + \pi_a^2 + \pi_b^2)$$

$$\frac{\delta^2 L}{\delta p \delta q} = \frac{\delta^2 L}{\delta q \delta p} = S\,(1 + \pi_b) + (\pi_a - \pi_b)\left(r + \frac{1}{r}\right)\left(s + \frac{1}{s}\right)$$

$$\frac{\delta^2 L}{\delta p \delta \pi_a} = \frac{\delta^2 L}{\delta \pi_a \delta p} = 2\left[Sp\pi_a + \frac{9}{2}\left(r + \frac{1}{r}\right)\left(s + \frac{1}{s}\right) - (18)\right]$$

$$\frac{\delta^2 L}{\delta p \delta \pi_b} = \frac{\delta^2 L}{\delta \pi_b \delta p} = 2\left[Sp\pi_b - \frac{9}{2}\left(r + \frac{1}{r}\right)\left(s + \frac{1}{s}\right) - (19) + 49\right]$$

$$\frac{\delta^2 L}{\delta p \delta r} = \frac{\delta^2 L}{\delta r \delta p} = q\,(\pi_a - \pi_b)\left(s + \frac{1}{s}\right)\left(1 - \frac{1}{r^2}\right)$$

$$\frac{\delta^2 L}{\delta p \delta s} = \frac{\delta^2 L}{\delta s \delta p} = q\,(\pi_a - \pi_b)\left(r + \frac{1}{r}\right)\left(1 - \frac{1}{s^2}\right)$$

$$\frac{\delta^2 L}{\delta q^2} = 16 - 2\left(r + \frac{1}{r}\right)\left(s + \frac{1}{s}\right) + \left(r^2 + \frac{1}{r^2}\right)\left(s^2 + \frac{1}{s^2}\right)$$

$$\frac{\delta^2 L}{\delta q \delta \pi_a} = \frac{\delta^2 L}{\delta \pi_a \delta q} = \left(r + \frac{1}{r}\right)\left(s + \frac{1}{s}\right) p$$

$$\frac{\delta^2 L}{\delta q \delta \pi_b} = \frac{\delta^2 L}{\delta \pi_b \delta q} = \left[8 - \left(r + \frac{1}{r}\right)\left(s + \frac{1}{s}\right)\right] p$$

$$\frac{\delta^2 L}{\delta q \delta r} = \frac{\delta^2 L}{\delta r \delta q} = s\,[(3) - (2)] + \frac{1}{s}[(7) - (6)] - \frac{s}{r^3}[(11) - (10)]$$
$$- \frac{1}{sr^2}[(15) - (14)] + p\,(\pi_a - \pi_b)\left(s + \frac{1}{s}\right)\left(1 - \frac{1}{r^3}\right)$$
$$+ 2q\left[\left(s^2 + \frac{1}{s^2}\right)\left(r - \frac{1}{r^3}\right) - \left(s + \frac{1}{s}\right)\left(1 - \frac{1}{r^2}\right)\right]$$

$$\frac{\delta^2 L}{\delta q \delta s} = \frac{\delta^2 L}{\delta s \delta q} = r\,[(3) - (2)] + \frac{1}{r}[(11) - (10)] - \frac{r}{s^2}[(7) - (6)]$$
$$- \frac{1}{rs^2}[(15) - (14)] + p\,(\pi_a - \pi_b)\left(r + \frac{1}{r}\right)\left(1 - \frac{1}{s^2}\right)$$
$$+ 2q\left[\left(r^2 + \frac{1}{r^2}\right)\left(s - \frac{1}{s^3}\right) - \left(r + \frac{1}{r}\right)\left(1 - \frac{1}{s^2}\right)\right]$$

$$\frac{\delta^2 L}{\delta \pi_a^2} = Sp^2$$

$$\frac{\delta^2 L}{\delta \pi_a \delta \pi_b} = \frac{\delta^2 L}{\delta \pi_b \delta \pi_a} = 0$$

$$\frac{\delta^2 L}{\delta \pi_a \delta r} = \frac{\delta^2 L}{\delta r \delta \pi_a} = pq\left(s + \frac{1}{s}\right)\left(1 - \frac{1}{r^2}\right)$$

$$\frac{\delta^2 L}{\delta \pi_a \delta s} = \frac{\delta^2 L}{\delta s \delta \pi_a} = pq\left(r + \frac{1}{r}\right)\left(1 - \frac{1}{s^2}\right)$$

$$\frac{\delta^2 L}{\delta \pi_b^2} = Sp^2$$

TABLE 4 (Concluded)

$$\frac{\delta^2 L}{\delta \pi_a \delta r} = \frac{\delta^2 L}{\delta r \delta \pi_a} = pq\left(s + \frac{1}{s}\right)\left(1 - \frac{1}{r^2}\right)$$

$$\frac{\delta^2 L}{\delta \pi_a \delta s} = \frac{\delta^2 L}{\delta s \delta \pi_a} = pq\left(r + \frac{1}{r}\right)\left(1 - \frac{1}{s^2}\right)$$

$$\frac{\delta^2 L}{\delta \pi_b^2} = Sp^2$$

$$\frac{\delta^2 L}{\delta r \delta \pi_b} = \frac{\delta^2 L}{\delta \pi_b \delta r} = -pq\left(s + \frac{1}{s}\right)\left(1 - \frac{1}{r^2}\right)$$

$$\frac{\delta^2 L}{\delta s \delta \pi_b} = \frac{\delta^2 L}{\delta \pi_b \delta s} = -pq\left(r + \frac{1}{r}\right)\left(1 - \frac{1}{s^2}\right)$$

$$\frac{\delta^2 L}{\delta r^2} = -\frac{2}{r^3}\left\{[pq(\pi_a - \pi_b) - q^2]\left(s + \frac{1}{s}\right) - sq[(10) - (11)]\right.$$
$$\left. - \frac{q}{s}[(14) - (15)]\right\} + q^2\left(s^2 + \frac{1}{s^2}\right)\left(1 + \frac{\dot{3}}{r^4}\right)$$

$$\frac{\delta^2 L}{\delta r \delta s} = \frac{\delta^2 L}{\delta s \delta r} = q\left\{[(3) - (2)] - \frac{1}{s^2}[(7) - (6)] + \frac{1}{r^3}[(10) - (11)]\right.$$
$$\left. - \frac{1}{r^2 s^2}[(14) - (15)]\right\} + pq(\pi_a - \pi_b)\left(1 - \frac{1}{r^2}\right)\left(1 - \frac{1}{s^2}\right)$$
$$- q^2\left[\left(1 - \frac{1}{s^2}\right)\left(1 - \frac{1}{r^2}\right) - 2\left(s - \frac{1}{s^3}\right)\left(r - \frac{1}{r^3}\right)\right]$$

$$\frac{\delta^2 L}{\delta s^2} = \frac{2}{s^3}\left\{[pq(\pi_a - \pi_b) - q^2]\left(r + \frac{1}{r}\right) - rq[(6) - (7)]\right.$$
$$\left. - \frac{q}{r}[(14) - (15)]\right\} + q^2\left(r^2 + \frac{1}{r^2}\right)\left(1 + \frac{3}{s^4}\right)$$

3. **One of r or s becomes imaginary.** Take the value $r = 1$ or $s = 1$ in place of the imaginary value. Proceed to estimate the other parameters as usual. This happens if there is an "interaction" between order and code bias effects.

4. **Both of r and s become imaginary.** Take both values as 1. It will make $q = 0$, as it should. Proceed for the other parameters as normally. Again, as in 3, the order and code biases are interacting (violating the multiplicativity assumption).

5. **One or both of r, s become infinite, due to zero denominator.** Change any *one* of the observed A votes or B votes to an E vote. This will bias the data slightly, not appreciably, however, unless the sample of judgments $4N$ is very small. It is for small $4N$ that this difficulty will arise, and the above step resolves the difficulty.

6. **Small negative estimate for q.** When the parameter q is zero its estimate can often arrive as negative. One may adopt two courses:

a) Keep the negative value of q and proceed to find p, π_a, π_b, π_e, as usual. These values will be quite normal.

b) Force q to be zero and obtain biassed estimates of the other parameters p, π_a, π_b, π_e. There does not appear to be any merit in this.

Large negative estimates for q indicate the model is invalid.

7. Slightly greater than 1 value for the estimate of q. Take q=1. There is nothing else to calculate. No one can discriminate, but everyone behaves as if they could. This could happen, for instance, when running a dummy-test with products A and B identical, big order effect, big code bias, and a forceful questionnaire.

Again, if the estimate of q is much greater than 1, suspect the model.

Numerical illustration. Suppose the observations in Table 5 below are obtained:

TABLE 5

Observed relative frequencies

Type of vote	A first A low	A first A high	A last A low	A last A high	Total
O	0.098	0.102	0.100	0.095	0.395
A	0.611	0.589	0.551	0.544	2.295
B	0.258	0.281	0.319	0.335	1.193
E	0.033	0.028	0.030	0.026	0.117
	1.000	1.000	1.000	1.000	4.000

Then

$$r^2 = \frac{611 + 281 - 258 - 589}{551 + 335 - 319 - 544} = 1.9565 \qquad r = 1.3987$$

$$s^2 = \frac{611 + 319 - 258 - 551}{589 + 335 - 281 - 544} = 1.2222 \qquad s = 1.1055$$

$$t = 2.1243 \qquad r^4 = 3.8275 \qquad rs = x = 1.5463 \qquad \frac{r}{s} = y = 1.2652$$

$$s^4 = 1.4935 \qquad x^3 = 3.6973 \qquad y^3 = 2.0252$$

$$x^4 = 5.7171 \qquad y^4 = 2.5623$$

$$\frac{s}{r} = 0.7904 \qquad \frac{1}{rs} = 0.6467$$

$$q = \frac{-1.102 \cdot 1.0621 + 0.353 \cdot 1.5463 + 0.308 \cdot 1.2652 + 0.209 \cdot 0.6467}{\frac{4.8275 \cdot 2.4935}{1.9564 \cdot 1.2222} - (2.1243)^2}$$

$$p = 1 - 0.1602 - \frac{1}{4}(0.395) = 0.7411$$

$$\pi_a = \frac{2.295 - 0.1602 \cdot 2.1243}{2.9644} = 0.6594$$

$$\pi_b = \frac{1.193 - 0.1602 \cdot 1.8757}{2.9644} = 0.3011$$

$$\pi_e = 0.0395 \qquad \pi_a - \pi_b = 0.3583 \qquad p(\pi_a - \pi_b) = 0.2655$$

$$\left.\begin{aligned} &qx^4 = +0.9159 \\ &[(3) - (2) + (\pi_a - \pi_b)p - q]x^3 = -0.9158 \\ &[(14) - (15) - (\pi_a - \pi_b)p + q]x = 0.1604 \\ &-q = -0.1602 \end{aligned}\right\} + 0.0003 \text{ (rounding error)}$$

$$\left.\begin{aligned} &qy = +0.4105 \\ &[(7) - (6) + (\pi_a - \pi_b)p - q]y = -0.4105 \\ &[(10) - (11) - (\pi_a - \pi_b)p + q]y = +0.1603 \\ &-q = -0.1602 \end{aligned}\right\} + 0.0001 \text{ (rounding error)}$$

CONCLUSIONS FOR NUMERICAL EXAMPLE

It is estimated that 74.11% of consumers can discriminate between products A and B. Of these 74.11% the majority, 48.87% prefer A, 22.31% prefer B, 2.93% have equal preference. There is an estimated 25.89% of consumers who cannot really discriminate, but 16.02% will behave as if they could when asked to participate in the type of test given, with the remaining 9.87% admitting in such tests their inability to discriminate.

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THE NONSPECIFICITY OF DIFFERENCES IN TASTE TESTING FOR PREFERENCE

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The purpose of this paper is to report a taste-preference experiment with raisins which emphasizes the distinction between difference testing and preference ratings based on organoleptic examinations of foods, beverages, perfumes and similar substances. The experiment involves simple, single-paired comparisons, because such procedures are fundamental in most ranking procedures.

It is sometimes assumed that products can be classed as better than similar products by mass testing that consists of having the products ranked according to preference by a great number of persons. The tables giving the number of favorable responses necessary to indicate statistical significance assume the same direction of preference for all testers and ability to detect the difference if any exists. These two assumptions will now be discussed in some detail, and applied to the experiment with raisins.

MASS CONSUMER PREFERENCE TESTING BASED ON ONE TEST PER CONSUMER

If none of the testers can distinguish a difference, then the products will be equally preferred and in a series of trials about half the time each will be scored over the other. The probabilities of each number of scorings of A over B, say, will be given by

$$\left(\frac{1}{2}+\frac{1}{2}\right)^n \qquad (1)$$

where n is the number of testers employed.

If the difference can be detected by each tester, but one-half the testers prefer A over B and one-half prefer B over A, and we consider a sample of n tasters drawn at random from such a population of testers, then the probabilities of each number of scorings of A over B will be again given by (1). That is, the two cases are indistinguishable on the basis of an experiment which simply records the preference for the two products.

If all preferences are in the same direction we have the usual one-tailed test *(5)* if the questions are: is A preferred to B or is B preferred to A, and the usual two-tailed test if it is merely asked if one product is preferred to another. The probabilities involved are either the right or left tails or both tails of (1). If the difference can be detected and part of the preferences are one way and part the other, say the proportion that prefer A is p and proportion that prefer B is q, and $p + q = 1$, then the probabilities of given numbers of A over B in preference expressions are given by:

$$(q + p)^n. \qquad (2)$$

The distribution (2) can be quite skewed either to the right or to the

left. If p can be estimated from a sample of n so that it can be determined that n_1 prefer A and n_2 prefer B, $n_1 + n_2 = n$ then (2) becomes

$$\left(\frac{n_1}{n} + \frac{n_2}{n}\right)^n \tag{3}$$

as an approximation.

In many cases the direction of preference may be difficult or impossible to determine so it may not be practical to find out if and to what extent one substance is preferred to another for more than one individual at a given time.

It has been shown by Baker *et al (1, 2)* and Mrak *et al (4)* that people vary over a wide range of the concentration of the stimulus in going from a zero value of the probability of detecting a difference in paired comparisons to a probability of 1 or certainty of detecting a difference. In particular in Baker *et al (1)*, it is shown that sucrose can vary from 0.017% to 0.548% as the number of people detecting a difference from distilled water increases from zero percent to 100%. The relation is essentially linear if concentration is measured on a logarithm to the base 2 scale. On this scale the concentration varies from 0.5 to 5.

The effect of the wide spread of partial ability to detect existing differences on the probability of overlooking real differences has been discussed in the papers cited. The implications for consumer testing for preference are discussed from the standpoint of the lack of ability of many consumers to detect differences that are important to others by Mrak *et al (4)*.

If we consider that direction of preference can vary among members of the testers or potential testers then the model of possible ratings observed in a preference experiment becomes even more complicated and in many cases the complete interpretation is impossible. The model could be further complicated by assuming that the strength of preference is a function of number of previous ratings.

For the purposes of illustration let us assume that we are investigating the preference of a population for A over B. Fifty percent of the population cannot detect the difference between A and B; 75% of those who can detect the difference between A and B prefer A and 25% prefer B to A. We wish to write out the probabilities for 0, 1, 2, . . . n expressed preferences for A. To specify still further let us take $n = 5$ and then numerous generalizations of this restricted case may become apparent.

In drawing a sample of five from our population we can get 0, 1, 2, 3, 4, 5 people who can detect the difference. The probabilities of getting these numbers of people are .0312, .01562, .3125, .3125, .1562, .0312, respectively. If we get no people who can detect the difference, the conditional probability distribution is simply $(\frac{1}{2} + \frac{1}{2})^5$. If we get one person who can detect the difference we have the following situation:

Number of preferences expressed for A

Weight	0	1	2	3	4	5
¾		.0625	.2500	.2750	.2500	.0625
¼	.0625	.2500	.3750	.2500	.0625	
Weighted total	.0156	.1094	.2812	.3438	.2031	.0469

where the first two rows are given as the terms of $(\frac{1}{2} + \frac{1}{2})^4$ and the third row is the weighted sum of the first two. From similar considerations we obtain the following results:

No. expressing preference for A	Probability conditional on getting specified no. of people who can detect the difference						Weighted Total
	0	1	2	3	4	5	
0	.0312	.0156	.0078	.0039	.0020	.0010	.0074
1	.1562	.1094	.0703	.0430	.0254	.0146	.0618
2	.3125	.2812	.2344	.1797	.1289	.0897	.2060
3	.3125	.3438	.3594	.3515	.3164	.2637	.3434
4	.1562	.2031	.2578	.3164	.3691	.3955	.2860
5	.0312	.0469	.0703	.1055	.1582	.2373	.0954
Weights	.0312	.1562	.3125	.3125	.1562	.0312	1.0000

In our sampled population pronounced preferences exist among the testers who can detect the difference. Some prefer a difference in one direction and some the reverse difference. The resulting probability distribution of 0, 1, 2, 3, 4, 5 expressed preferences is very similar, however, to that obtained under the null hypothesis of no preference. If we operate under the critical region of 0 or 5 preferences for A we are operating at the 6% level for the probability of saying a preference exists where in fact there is no such preference. The probability of a type II error if our complicated population is really being sampled is 0.90. That is, there is little possibility of separating the two situations by such sampling.

The generalization of our tables of conditional probabilities is obvious and it is easy but tedious to explore any desired situation.

MASS CONSUMER PREFERENCE TESTING BASED ON MULTIPLE TESTS PER CONSUMER

If we wish to plunge deeper into the turbid mess of consumer preference testing detailed above, it is necessary to test each consumer more than once. One method of procedure would be to define a universe that is realistic. Take a random sample of n consumers, or take random samples of $n_i (i = 1, 2, 3, \ldots k)$ from k well defined strata of the universe. Then consider that each person selected will be tested more than once, although all need not necessarily be tested the same number of times.

Ferris *(3)* has considered a k-visit method of consumer testing based on a simple, somewhat unrealistic model for testing the preference for A over B. He defines 3 proportions of the sampled population universe as follows:

π_a: prefer A

π_b: prefer B

π_o: cannot distinguish A from B or have no preference for A over B.

No allowance is made in this model for consumers who can sometimes distinguish A from B or might sometimes prefer A over B. The point is, as mentioned previously, that the probability of distinguishing A from B for any one person or any group of persons increases from zero to one over a rather wide range for the difference between A and B. There is also the

possibility of change in preference as the testing proceeds. This latter situation could be guarded against only by rather elaborate techniques.

One method of proceeding is to test each of the n consumers of the random sample once and record the stated preference. Consumers should be forced to state a preference because of subliminal considerations. Then test each again. Those who reverse their preferences are temporarily sorted out. Similar steps are completed with reversals sorted out until say 5 consistent statements are made concerning the preference for A or B. Such people are likely to have a real preference for A or a real preference for B as the case may be. If very few prefer A or very few prefer B we conclude that the preference is essentially in one direction and this is a very valuable thing to know. If the numbers preferring A and B are both small, we can conclude there is no difference in preference. Also at this stage we know what proportion of people can consistently tell the difference between A and B and of these consumers we can estimate what proportion prefer A and what proportion prefer B.

In choosing 5 consistent judgments we are operating at about the 3% level.

Now we can either lump all other consumers in the same category or we can try to sort them into further categories.

Thus, of those sorted out at the first reversal, we can start re-running up to a proper number of trials but sorting out all those who show two changes. To attain the 3% level previously settled on, we can stop after 8 tests with only one change in stated preference.

If we permit 2 changes in stated preference we should have 11 tests. If we permit 3 changes we should have 16 tests and so on.

The number of consumers in each of these categories give estimates of the proportion of the population which tend, in varying degrees, to prefer A or to prefer B. These proportions could be smoothed in various ways to give improved estimates of the proportions.

Chi-square is available for testing an observed structure against any hypothetical or other observed structure.

RAISIN EXPERIMENT

In order to explain the applicability of the foregoing ideas in the interpretation of actual consumer preference investigations, we designed and carried out an experiment with raisins.[6] One type of ordinary sundried raisins was designated as untreated (U), whereas the other raisins were treated by dipping in a soda solution and dehydrating, and were designated as treated (T).

The score sheets included instructions for the tasters and an example of the scoring procedure. For each of the 10 days of tasting, the tasters were to circle the number of the paired sample they preferred, circle whether they preferred it slightly or very much, and write in the actual date and time the samples were tasted.

The numbering, coding, and keying of the score sheets was done in such a way as to eliminate as much sampling and technique bias as possible.

From the 404 kits sent out, 211 questionnaires were returned in completed, usable form. Thus our sampled population can not be considered the general university student population, but is that segment of the students sufficiently interested and reliable to cooperate in the study on a voluntary basis. The results are not designed to reveal possible biases introduced by incomplete reporting, but some groups did have more

[6] Made available to us by the California Raisin Advisory Board, Fresno, California.

TABLE 1

Number of males and females preferring treated raisins a stated number of times out of ten tastings. Null hypothesis frequencies are given for comparison with the total males and females

Number of times T preferred	Male	Female	Total	Null Hypothesis
0	14	37	51	0.2
1	9	16	25	2.1
2	8	14	22	9.3
3	4	9	13	24.7
4	10	9	19	43.3
5	8	2	10	51.8
6	8	6	14	43.3
7	8	5	13	24.7
8	5	4	9	9.3
9	13	8	21	2.1
10	9	5	14	0.2
Total	96	115	211	211

Note: Testing for sex difference, we find chi-square = 21.18 with 10 degrees of freedom and hence P = 0.02. However, much of the value of chi-square is due to the 0 — times preferred class and we do not consider that this one class difference indicates a real difference in ability to distinguish between raisins. It may be that the attitudes of the two sexes are slightly different.

complete returns than others. Comparisons among the groups did not reveal any pronounced trends or discrepancies.

Table 1 gives a breakdown of the data by male and female for the number of times that T was preferred over U. Since it is doubtful that sex is important as far as preferring the two kinds of raisins is concerned, the total of two sexes is compared with the number of those expected under the null hypothesis, that is, under the assumption that there is no preference for either the treated or untreated. It is seen that both tails are very heavy, indicating that some people preferred U and some T. Table 2 gives the numbers of testers that have been consistent in their preferences for all trials up to and including the first, second and so on to the tenth trial. We note that 10 trials eliminated 67 testers from the original 81 for the people preferring treated raisins, and that 79 were eliminated from 130 for the testers preferring untreated raisins. The percentages are quite different in the two cases, but the actual number eliminated are essentially the same, which seems reasonable, in that those who could not tell the difference, or were indifferent to the difference, would contaminate both preference groups in about the same numbers in the beginning.

We can construct a hypothetical model that will approximately represent the total column in Table 1. We shall assume 3 binomial distributions. The first component

TABLE 2

Numbers who consistently preferred T and U raisins for one to ten trials

Trial number	Number preferring T every time	Number preferring U every time
1	81	130
2	51	110
3	36	98
4	24	80
5	20	73
6	19	69
7	17	64
8	15	61
9	15	56
10	14	51

distribution will represent those people who prefer untreated raisins, the second component will represent those testers who can not tell the difference or are indifferent to it, and the third component will represent the people who prefer the treated raisins.

The p value for the second component is 0.50. We estimate p for the first component to be 0.07, and for the third component to be 0.90. These values are not zero and one, respectively, for two reasons. Even people with definite preferences may make occasional errors in recording or judgment and, also, while most people in the extreme groups may always be able to tell the difference, there may be other people in the groups who can only distinguish between the raisins part of the time.

We have to estimate the number of testers in each component. We estimate 100 for the first, 64 for the second, and 47 for the third. The values for the p's and numbers in the component were arrived at by numerical methods. Estimating equations in terms of moments were written down. However, the equations are non-linear and can only be solved by methods of successive approximations.

There are 11 classes, and we estimate 5 constants. This leaves 6 degrees of freedom for a chi-square test of goodness-of-fit. Table 3 gives the comparison between the

TABLE 3

Comparison of observed numbers of testers consistent for 1 to 10 trials with the theoretical model $100(0.93+0.07)^{10}+64(0.50+0.50)^{10}+47(0.10+0.90)^{10}$

Number of times T raisins preferred	Observed number of testers	Theoretical number of testers
0	51	48.64
1	25	37.07
2	22	15.09
3	13	9.98
4	19	13.46
5	10	15.76
6	14	13.66
7	13	10.20
8	9	11.85
9	21	18.84
10	14	16.45
Total	211	211.00

Chi-square = 15.37. $P = 0.02$.

observed data for the numbers preferring treated raisins 0 to 10 times and our theoretical model,

$$100(0.93+0.7)^{10}+64(0.5+0.5)^{10}+47(0.10+0.90)^{10}.$$

The main inconsistencies between observation and theory are for the numbers of testers preferring treated raisins 1 and 2 times. This may be a vagary of chance ($P = 0.02$).

On the basis of Table 3, we estimate that our 211 tasters included 100 who definitely preferred untreated raisins, 47 that preferred treated raisins, and 64 who could not tell the difference, or were indifferent to it.

SUMMARY

Attention has been called to the confusion that may arise in consumer preference testing due to difference in direction of preference and subliminal considerations. An operationally meaningful method of structuring a definite universe of potential consumers has been given with a suggestion as to how to test an observed structure against any hypothetical or other observed structure.

To illustrate the methods and considerations suggested a raisin experiment is reported and analyzed in detail. In this case we had all testers taste the same number of times; namely, ten.

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